Mahder Seifu Manenda

Graduated in

Erasmus Mundus Masters In Membrane Engineering

Title

Separation of Bioactive Peptides by Electrodialysis with Ultrafiltration Membranes: Membrane Characteristics, Ex-situ and In-situ Digestion and Their Impact on Peptide Migration

Dissertation for obtaining the Master degree in Membrane Engineering

Erasmus Mundus Master in Membrane Engineering

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"In questions of science, the authority of a thousand is not worth the humble reasoning of a single individual." Galileo Galilei

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ABSTRACT

Trypsic hydrolysis of whey protein isolate was performed simultaneously with (in-situ) and before (ex-situ) fractionation by electrodialysis with ultrafiltration membrane (EDUF) to obtain bioactive peptides. Two ultrafiltration membrane (UFM) materials, PES and PVDF, were used for a 120 minute EDUF fractionation of the hydrolysate. The two membranes showed similar zeta potential measurements at the pH of operation, 7.8, but had significantly different conductivity which decreased significantly after use in EDUF. Peptide migration to anionic (A^-_{RC}) and cationic (C^+_{RC}) peptide recovery compartment was strongly dependent on the electrical conductivity of the UFMs than their types or the digestion strategy used. For UFMs with close values of conductivity, peptide migration to the A^-_{RC} was observed to be higher with in-situ digestion while peptide migration to the C^+_{RC} was higher in an ex-situ digestion. When the two membrane types, PES and PVDF, have closer values of conductivities, PVDF was observed to exhibit more migration than PES. Peptide migration to the C^+_{RC} varied from 16.56 ± 5.36 μg/mL to 103.10 ± 2.76 μg/mL for PVDF membrane with significantly different conductivities: 2.73 ± 0.32 mS/cm and 5.47 ± 0.56 mS/cm, respectively. Peptide migration to the A^-_{RC} varied from 4.41 ± 0.86 μg/mL to 49.65 ± 6.13 μg/ml for PVDF membrane with significantly different conductivities: 3.163 ± 0.12 mS/cm and 5.23 ± 0.04 mS/cm, respectively. HPLC-MS studies showed 23 major peaks that were generated on whey protein isolate digestion by trypsin. 9 of these peaks migrated to the A^-_{RC} while 3 migrated to the C^+_{RC}. Among the anionic peptides 3 peptides are known to have hypocholesterolemic effect, 1 is antibacterial and 1 is antihypertensive. Among the cationic peptides 1 is antihypertensive. EDUF appears to be a powerful new technique that can fractionate bioactive peptides in terms of mass and charge.

Keywords: Hydrolysis, Bioactive Peptides, Peptide Migration, Electrodialysis, Ultrafiltration, Nutraceuticals

Index of Figures

Index of Tables

Abbreviations

EDUF: electrodialysis with ultrafiltration membranes

ED: electrodialysis

UFM: Ultrafiltration Membrane

UF: Ultrafiltration, NF: Nanofiltration

PVDF: Polyvinylidiene Floride

PES: Polyether Sulfone

MWCO: Molecular Weight Cutoff

MW: Molecular Weight

ACE: Angiotensin Converting Enzyme

BCA: Bicinchoninic Acid

IEM: Ion-Exchange Membrane

CEM: Cation-Exchange Membrane.

AEM: Anion-Exchange Membrane

A^-_{RC}: Anionic Peptides Recovery Compartment;

C^+_{RC}: Cationic Peptides Recovery Compartment

pI: isoelectric point, WPI: Whey Protein Isolate

TFA: Trifluoroacetic Acid

TABLE OF CONTENTS

1. Introduction

1.1. Background and Motivation

As the knowledge of molecules contained in food sources deepen, scientists have long started looking at food as more than just a source of calories and nutrients. The presence of bioactive molecules contained in the complex food matrix and other natural sources has attracted much attention from food scientists and the food industry. Bioactive molecules are molecules obtained from natural sources that can exert physiological changes in microbes or in humans. They can exist as polysaccharides or oligosaccharides, polyphenolic compounds, conjugated linoleic acids, peptides etc. The existence of such molecules in everyday food sources, making them functional foods, or the isolation of such molecules from food sources and their preparation into specialized products (nutraceuticals) can be a cost effective alternative to the increasingly very expensive drug based health care (Health Canada, 2013).

Bioactive peptides have long been known to exert a range of physiological effect in humans and other organisms acting as antihypertensive, antithrombotic, antimicrobial, antioxidative, immunomodulatory and opioid molecules (Bazinet L & Firdaous L, 2009). The bioactivity of these peptides is inherent in their amino acid sequence that sometimes leads to multi-functionality. However due to their low concentrations in nature and their interactions with other molecules that can potentially lead to loss of bioactivity, bioactive compounds have limited bioavailability. These led to several attempts to either directly synthesize the peptide sequences or isolate them from their natural sources efficiently. Direct synthesis by chemical method or using recombinant DNA technology are, as of now, costly for large peptides and difficult to streamline while isolation from natural sources including everyday food materials by hydrolysis and then isolation presents a cost effective and easy to scale up alternative.

Hydrolysis of milk/whey proteins by digestive enzymes including trypsin have been reported to be an important source of bioactive peptides (Korhonen H. & Pihlanto A., 2003). Isolation of these peptides to obtain specific function is a challenge to food scientists and dairy industries. Membranes processes and materials play an important role in the isolation of bioactive peptides obtained from enzymatic hydrolysis of milk/whey protein. An enzymatic membrane reactor (EMR), for instance, usually uses ultrafiltration membranes (UFMs) to immobilize the enzyme and also to separate the digested products (Kitts D. and Weiler K. 2003). Pressure driven process like ultrafiltration (UF) and nanofiltration (NF) have also been used, separately or in combination, to fractionate bioactive peptides mainly by their mass and, to an extent, by their flowing charge interaction with NF membranes (Bazinet L & Firdaous L., 2009). In both cases the extent of fouling presents significant challenge and both lack the ability to simultaneously

fractionate cationic and anionic peptide which may have close molecular mass but completely different bioactivity (Yuanhui *et al*.2007).

Electromembrane processes; including electrically-enhanced membrane filtration systems, forced flow membrane electrophoresis and electrodialysis with ultrafiltration (EDUF) have the ability to fractionate cationic and anionic peptides even when the peptides have very close molecular weights. Moreover EDUF have the general advantage of lower fouling compared to purely pressure driven process (Langevin M., *et al* 2012). EDUF has now been used to isolate bioactive compounds from multitude of sources. Labbé *et al* (2005) used EDUF system to obtain a migration of 50% catechins (antioxidant molecules from a green tea infusion). Firdaous *et al* (2010) reported the isolation of an angiotensin converting enzyme (ACE) inhibitor peptide fraction from alfalfa white protein and Doyen *et al* (2012) used EDUF to recover and concentrate the active antibacterial fraction from a snow crab by-products hydrolysate.

In a recent study Doyen *et al* (2011) compared polyether sulfone (PES) and cellulose acetate (CA) membranes and reported no significant difference in total peptide migrations. The two membranes were reported to show a slight difference in the way their conductivities changed after being used in EDUF: PES membranes had reduced conductivity while CA did not. The authors reported no migration to the cationic recirculation compartment (C^-_{RC}) for both membranes until after 180 minutes of EDUF. This could be due to the 20kDa MWCO of UFM used and also due to a relatively smaller electric field strength applied: 2V/cm. Hence we propose to apply larger electric field strength and use UFMs with a larger MWCO to enhance peptide migration and further study the role of UFM material using PES and PVDF membrane materials. Moreover the effect of digestion within the EDUF setup or outside of it has not been studied before. PES and PVDF membranes have desirable physical and chemical properties that make them popular for ultrafiltration and other applications (Rong G. *et al.* 2005, Zhang Q. *et al.* 2002, & Huyen T. *et al.*2006). Their differences in surface properties and electrical properties resulting from their chemical makeup could be important factors in EDUF.

1.2. Objectives

In light of the background presented above, the aim of the present work is investigate the role of digestion techniques and membranes in the separation of bioactive peptides from hydrolysate of whey protein isolate digested by trypsin using EDUF. The specific objectives include:

i. to study the effect of UFM material on EDUF isolation of bioactive peptides
ii. to study the effect of in-situ and ex-situ digestion on EDUF isolation of bioactive peptides
iii. to characterize the peptides generated and use data from the literature to identify their respective potential bioactivity

2. Literature Review

2.1. Bioactive Peptides

2.1.1. Definitions

Bioactive peptides are generally defined as specific protein fragments that have a positive impact on body functions and conditions and may ultimately influence health (Bazinet L & Firdaous L, 2009). Even though the terms “functional foods” and nutraceuticals have now become part of the scientific lexicon in food science literature, a universal definition for both is difficult to find. However, functional foods can be defined as products that are consumed as food and not in dosage form and have beneficial effect on the health of the consumer. Nutraceutical is defined, in Canadian law, as referring to "a product isolated or purified from foods that is generally sold in medicinal forms not usually associated with food. A nutraceuticals are demonstrated to have physiological benefits or provide protection against chronic diseases” (Health Canada, 2013).

2.1.2. Bioactive Peptides from Milk

Bioactive molecules occur naturally in plant and animal products, usually at very low concentrations. Several bioactive peptides that are present in many food proteins have long been demonstrated to posses physiological benefits (Kitts D. & Weiler K., 2003) apart from the provision of basic nutrition as there is more to food than just calories and nutrients. Given their benefits it is not surprising to see the number of publication regarding functional foods and nutraceuticals from protein sources increasing by the year and the global functional foods and nutraceutical market growing at a rate that is outpacing the processed food market (Korhonen H. & Pihlanto A., 2003).

Proteins have been identified as a source of several physiologically active peptides which act in a variety of ways. Some of these peptides have the potential to inhibit the growth of micro-organisms (anti-microbial activity), interact with opioid receptors in the brain and have agonistic or antagonistic activities (act as opiate), prevent internal clotting of blood (antithrombotic activity) or prevent the complications of high blood pressure (anti-hypertensive activity), and alter the body's immune response (immunomodulation) etc (Kitts D. & Weiler K., 2003). Such a wide range of bio-activity in these peptides has generated much attention to use their sources as functional foods and the peptides as nutraceuticals in the food industry and research. In an age where the cost of discovering new drugs is skyrocketing, the use of everyday food nutrients for the generation of active ingredients is becoming very attractive in populations which are aging and also in populations with limited resources to pay for expensive medications. In this regard development of functional foods and nutraceuticals can be utilized to

improve the human health, reduce health care costs and support economic development in rural communities (Korhonen H. & Pihlanto A., 2003).

Milk proteins are considered the most important source of bioactive peptides. The activity of these peptides is based on their inherent amino acid composition and sequence found buried in the native protein. The size of active sequences in peptides may vary from 2 – 20 amino acid residues, and many peptides are known to reveal multifunctional properties (Kitts D. & Weiler K., 2003). Over the last decade a great number of peptide sequences with different bioactivities have been identified in various milk proteins. The best characterized sequences include antihypertensive, antithrombotic, antimicrobial, antioxidative, immunomodulatory, and opioid peptides (Korhonen H. & Pihlanto A., 2003). These peptides have been found in enzymatic protein hydrolysates and fermented dairy products, but they can also be released during gastrointestinal digestion of proteins, as reviewed in many articles (Mullaly M. *et al* 19975, Vandekerckhove J *et al* &7). For instance trypsic digestion can release peptides β-LG f15– 20, β-LG f102–105 and β-LG f142–148 all of which are identified as having ACE inhibitory activity (Mullaly, M. *et al* 1978, Fitzgerald R. & Meisel, H., 2009). Trypsin also releases bactericidal peptides β-LG f15–20 and β-LG f92–100 (Pellegrini, A. *et al*, 2000), and hypocholesterolemic peptide β-LG f71–75 (Nagaoka, S, *et al* 2001), and the opioid peptide from β-LG f102–105 (Meisel, H. & Schlimme E., 1995).

2.2. Methods of Bioactive Peptide Production and Separation

2.2.1. Production of Bioactive Peptides

2.2.1.1. Direct Peptide synthesis and Recombinant DNA Technology

Due to their low concentrations in nature and their interactions with other molecules that can potentially lead to loss of bioactivity, bioactive compounds have limited bioavailability. This challenge on bioavailability has encouraged scientists and industries to investigate possible synthesis, isolation, concentration and delivery of specific bioactive peptides for increased efficacy in their role as modulators of physiological functions. Direct peptide synthesis of specific sequences is one of the methods being investigated to produce bioactive peptides of desired amino acid sequence in the laboratory. This can be done by a liquid-phase or a solid-phase synthesis. The liquid-phase synthesis has been used for the synthesis of small peptides and its main advantage is that the intermediate products can be isolated and purified after each step of synthesis and recombined later to obtain larger peptides of the desired sequence. But the productivity of these methods is very limited except for relatively shorter sequences (8-10 amino acid) (Nishiuchi *et al*. 1998) Solid-phase peptide synthesis consists in the elongation of a peptide chain anchored to a solid matrix by successive additions of amino acids which are

linked by amide (peptide) bond formation between the carboxyl group of the incoming amino acid and the amino group of the amino acid previously bound to the matrix, until the peptide of the desired sequence and length has been synthesized (Nilsson *et al.* 2005). Despite their wide spread use in the pharmaceutical industry application of solid phase peptide synthesis for peptides of more than 10 residues are challenging and technically complicated (Patarroyo *et al*., 1988).

Recombinant DNA technology is also another method of producing desired peptides and if the system is established, peptides can be obtained in large quantities from very inexpensive starting materials via fermentation but this requires a long and expensive research and development phase before starting to produce the desired peptide (Korhonen , H. & Pihlanto A., 2003). In fact Meister *et al* (2003) produced β-Casomorphins (an opiate peptide from milk), from both human and bovine milk, by genetically engineering the genes for the peptide and cloning it into a plasmid of *E. coli* but the concentrations they obtained were very low (38.22 nmol/L). Hence the direct synthesis of peptides, chemically (for longer sequences than 8-10 amino acids) or by recombinant DNA technology, is both costly and technically complicated for scale up.

2.2.1.2. Microbial Fermentation

Microbial fermentation of food matrices and/or biomass can be a source of several bioactive peptides. A mixture of system of lactic acid bacteria, such as *Lactococcus lactis*, *Lactobacillus helveticus* and *Lactobacillus delbrueckii* var. *bulgaricus*, (Hartmann R. & Meisel H., 2007) have the enzymatic machinery to systematically cleave large proteins into oligopeptides and then into amino acids and also into bioactive peptides which can be extracted from the fermentation broth by various techniques. The enzymes involved in milk protein degradation can either be cell-envelope proteinases (CEP) or intracellular peptidases (Mullaly M. & Meisel H., 1997). Longer oligopeptides which are not transported into the cells can be sources for the liberation of bioactive peptides in fermented milk products when further degraded by intracellular peptidases after bacterial cell lysis or in the digestive system of humans. Yamamoto *et al.* (1994) reported that casein hydrolyzed by the cell wall-associated proteinase from *L. helveticus* CP790 showed antihypertensive activity in rats. Several ACE inhibitory and one antihypertensive peptide were isolated from the hydrolysate. Using the same proteinase, Maeno *et al.* (1996) identified a β-casein-derived antihypertensive peptide from the casein hydrolysate.

2.2.1.3. Enzymatic Digestion

Enzymatic digestion of peptides involves the release of peptide ad amino acids by hydrolysis of the peptide bond to obtain a hydrolysate. Hydrolysate's peptide composition, and consequently their properties, is dependent of protein and enzyme used, as well as, on hydrolysis conditions (temperature, pH, enzyme to substrate ratio and reaction time). Hydrolysates can be characterized according to several molecular characterization methods, including HPLC-MS, which reflect their molecular properties.

Enzymatic digestion of proteins by a digestive enzyme, or a combination of them, is the most common way of producing bioactive peptides from food materials. Enzymes like trypsin, chymotrypsin, alcatase, pepsin etc have been used to generate bioactive peptides from proteins. Amongst the enzymes, trypsin is the most commonly used mainly because of its tendency to generate more bioactive peptides (specially angiotensin-converting enzyme (ACE) inhibitor peptides) without needing to be combined with other enzymes (Meister W. *et al*., 1994). Trypsin digests proteins from milk that remain soluble at pH 4.6 which are commonly known as whey protein isolates (WPI). These proteins are commonly isolated by ion exchange chromatography or microfiltration and mainly contain β-lactoglobulin and α-lactalbumin both of which have high nutritional value. WPI also contain relatively high branched chain amino acids (leucine, isoleucine, valine) that contribute to their important role in muscle growth and repair. They are also an important source for bioactive peptides (Clare & Swaisgood, 2000) after trypsic digestion. However after digestion the peptides have to be separated in order to enhance their biological activity.

2.2.2. Separation of Bioactive Peptides

Membrane processes are the most commonly used processes used in a large scale separation of bioactive peptides.

2.2.2.1. Pressure Driven Processes in Bioactive Peptide Isolation

Membranes materials and processes play an important role both for the production and also isolation of bioactive peptides from the digestion of proteins. Enzymatic membrane reactor (EMR) consists of a coupling of a membrane separation process with an enzymatic reaction. It have been used for the continuous production and separation of specific peptide sequences by means a selective membranes which is used to immobilize the biocatalyst and also to separate the peptides fractionations. Ultrafiltration (UF) membrane bioreactors for example have been used for a simultaneous hydrolysis and isolation of bioactive peptides from a large variety of source proteins. Operation of such membrane bioreactors in the continuous mode has the

advantage of recycling the enzyme and continuous exposure of the protein to the digestive enzyme leading to an improved production of the peptides. Fractions of peptides with a wide range of molecular size and sequence can be obtained depending on the molecular cutoff of the ultrafiltration membrane used (Langevin M. *et al.* 2012). Ultrafiltration membranes have also been applied in a pressure drive process without involving immobilized enzymes to separate peptides from hydrolysates obtained from a separate digestion step.

In an interesting application of ultrafiltration membranes for the fractionation of hydrolysate obtained from whey proteins, Turgeon *et al* (Turgeon L. *et al.* 1990) used a stepped ultrafiltration procedure to limit the range of peptides obtained in different fractions to a narrow range. In their experiments they used ultrafiltration membranes with MWCO 1 and 30 kDa to enrich ACE inhibitory peptides from purified α- lactoglobulin and β-lactoglobulin and obtained two fractions: one rich in higher molecular weight peptides and the other rich in amino acids and low molecular weight peptides. Several studies have also reported the use of stepped ultrafiltration procedure to fractionate different hydrolysates in to different fractions which have different physiological effects depending upon the peptides they contain (Xie Z, *et al*., 2008, Pihlanto A. *et al*., 2008). But such procedures are limited because they use only the size of the peptides generated to fractionate the peptides as opposed to other emerging procedures which use a combination of size, shape and charge of peptides to fractionate them.

Several nanofiltration (NF) membranes were also used to separate peptides on the basis of charge interaction with the membranes since most peptides contain charged functional groups at a given pH. Tsuru *et al.* (1994) reported that separation of amino acids and peptides with nanofiltration membranes having a MWCO around 2000–3000 Da was successful based on a charge effect mechanism while nanofiltration membranes having a MWCO below 300Da were not suitable for separation of amino acids or peptides. Charged amino acids and peptides were rejected in their fractionation while neutral amino acids and peptides permeated through the FilmTech, Toray and Nitto membranes they used. The separation of peptides having different isoelectric points with nanofiltration membranes was possible by adjusting the pH (Tsuru *et al*. 1994).

The combination of membrane processes (UF and NF) is often used to separation of peptides. The first step of these processes consists in the UF of the hydrolysate in order to obtain rejection of the intact proteins and intermediate peptides. The resulting permeate fractions is then subjected to a fractionation by NF and a peptide fraction having a molar mass usually lower than 1 kDa is isolated by the charge interaction between the membrane and the peptide dictated by the nature of the membrane and pH of the solution. For instance, Butylina *et al* (2006) used a

combination of ultrafiltration and nanofiltration to fractionate peptides contained in sweet whey. In this combination the first step consisted in the ultrafiltration of the sweet whey with a MWCO of 10 kDa to completely retain the whey proteins. The resulting permeate fractions were then subjected to a fractionation by nanofiltration with a MWCO of 1 kDa. Amongst the pressure-driven membrane techniques, UF and NF are preferred for the fractionation of protein hydrolysates due to the fact that the molecular weight of most bioactive peptides is within the normal range of the MWCO of these membranes.

Despite their wide use in the fractionation of protein hydrolysates pressure-driven processes like NF and UF lead to a significant accumulation of particles on membrane leading to fouling that causes modification of the membrane transport selectivity (Langevin M. *et al.,* 2012) Moreover both UF and NF lack the ability to fractionate peptides depend on the charge of the peptide. Though in nanofiltration the charge of the peptide does play a role in determining its interaction with the membrane this role is limited as it doesn't allow simultaneous separation of oppositely charged peptides.

2.2.2.2. Electromembrane Processes in Bioactive Peptide Isolation

The challenge of significant fouling and the fractionation of peptides only by their molecular weight (not considering their charge and its change with pH) have made scientists to look for other options of bioactive peptide isolation. Electromembrane processes are among the options investigated for possible improvement of peptide isolation. These processes involve the coupling of the transport of electrical charges and a mass transfer across ion-exchange, bipolar, filtration etc membranes. These processes can be used together with pressure driven processes to reap the benefits of both types of process and improve process productivity.

In an interesting approach of combining pressure driven process with electromembrane processes Huotari *et al.* (1999) superimposed an electrical field to a conventional membrane filtration unit effectively forming an electrically-enhanced membrane filtration system. The superimposition can be either in a parallel orientation with flat sheet filtration membranes or the electric field can be applied between the inner rode and the membrane in a tubular membrane module. Loose nanofiltration and ultrafiltration membranes have been reported to be used in this configuration (Lapointe J. *et al.,* 2006). But this configuration lacks a distinct electrolyte recirculation compartment that could prevent pH changes caused by electrolysis of water at the electrodes. Moreover bioactive molecules are vulnerable to oxidation resulting from contact with the electrodes or oxidized species (Bazinet L. & Firdaous L., 2009).

In the recent years **Electrodialysis with Ultrafiltration Membranes (EDUF)** stacked has emerged to be capable of separating peptides not only on the basis of their molecular weight but also on the basis of their charge. In a configuration patented by Bazinet *et al.* (2005) EDUF consists of four recirculation compartment for the feed, anionic peptide recirculation compartment (A^-_{RC}), cationic peptide recirculation compartment C^+_{RC}, and the electrolyte solution at the anode and cathode (Figure 1). The feed recirculation is separated from A^-_{RC} and C^+_{RC} by two ultrafiltration membranes (UFMs) which impart the configuration the ability to screen peptides in terms of their molecular weights. Anion exchange membrane (AEM) and cation exchange membranes (CEM) separate the two peptide recovery compartments from the electrolyte recirculation compartment near the anode and cathode, respectively. The two UFMs can have the same or different MWCO, they can be of the same or different material, and interestingly as much as 7 UFMs have been placed in between the ion exchange membranes (IEMs) (Firdaous *et al*., 2010) giving an efficient functional unit. It is important to note that no pressure difference is applied in between any compartment of the EDUF configuration.

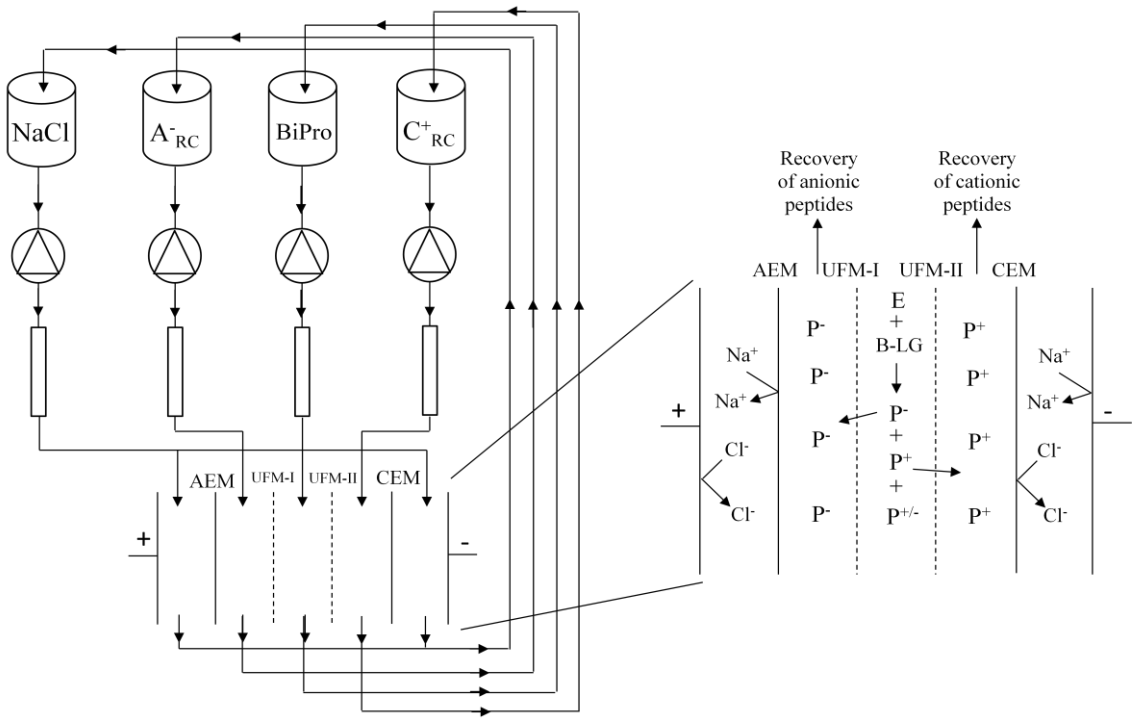

Figure 2.1: EDUF cell configuration for the hydrolysis of BiPro protein and recovery of anionic and cationic peptides. AEM: anion-exchange membrane, CEM: cation exchange membrane, UFM: ultrafiltration membrane, A^-_{RC}: anionic peptide recovery compartment, C^+_{RC}: cationic peptide recovery compartment, BiPro: whey protein extract, E: trypsin enzyme, P^+: cationic peptide, P^-: anionic peptide (Adapted from Doyen *et al*., 2013)

EDUF technology showed several potential applications for the food industry, notably for the separation and recovery of bioactive compounds from diverse raw matrices. Doyen *et al*., (2012) used EDUF to recover and concentrate the active antibacterial fraction from a snow crab by-products hydrolysate. They used two ultrafiltration membranes with different MWCO (20 kDa and 50 kDa) and two electrical field strengths (2 and 14 V/cm) and reported 94% abundance of peptides with molecular weight range 300–600 Da peptide in the recovery compartments. In a different report (Doyen *et al.,* 2011) the same researchers identified an anticancer peptide fraction from snow crab byproduct hydrolysate after a selective separation by EDUF with 20 kDa MWCO cellulose acetate (CA) UFM stacked in the system.

Firdaous *et al.,* (2010) reported the isolation of an angiotensin converting enzyme (ACE) inhibitor peptide fraction from alfalfa white protein hydrolysate by stacking a polyether sulfone (PES) UFM with10 kDa MWCO in EDUF cell. Poulin *et al.* (2006) performed fractionation of a β-lactoglobulin hydrolysate and demonstrated the simultaneous separation of acid and basic bioactive peptides is possible by stacking 20 kDa MWCO cellulose acetate (CA) UFMs. Roblet *et al.,* (2013) found out that pH modulation appeared to be an efficient way to concentrate the low molecular weight peptides (400 Da) in the cationic peptides recovery compartment C^{+}_{RC} and to limit the diversity of peptides recovered in the A^{-}_{RC} in purification of soy peptides from a complex matrix.

It is important to note that EDUF can indeed be used in isolation of bioactive molecules not only from protein sources but also from other several types of sources. Labbé *et al.* (2005) demonstrated that catechins (antioxidant molecules from a green tea infusion) can migrate at a high rate through an EDUF system. They reported that UFM with 1kDa can achieve migration as high as 50%. In a more recent study, Bazinet *et al.,* (2009) reported an 18% increase of the antioxidant capacity of cranberry juice the enriched by EDUF treatments demonstrating that the EDUF process might be used for natural enrichment of cranberry juice with antioxidant phenolics (Bazinet L. *et al.* 2009).

Despite the growing interest in EDUF and the parameters that influence separation in EDUF the effect of ultrafiltration membrane (UFM) material have not been studied extensively. In a comparative study Doyen *et al.* (2011) compared two different types of membranes, polyether sulfone (PES) and cellulose acetate (CA) for their performance when used in EDUF system. No significant difference in total peptide migration was reported between the two types of UFM material studied during EDUF separations. Total peptide migration depended only on the duration of EDUF and, for the C^{-}_{RC}, the authors observed no migration for both membranes until after 180 minutes of EDUF. PES membranes showed difference in conductivity after being used

in EDUF when compared to their respective control membranes but no significant such difference was detected for CA (Doyen A. *et al.*, 2011). In general the report indicates that CA UFM allowed the recovery of high molecular weight molecules (900-20000 Da) in both recovery compartments.

Polyethersulfone (PES) is a heat-resistant, transparent, amber, non-crystalline engineering plastic while PVDF is a highly non-reactive and pure thermoplastic fluoropolymer produced by the polymerization of vinylidene fluoride. PVDF highly desirable insolubility and electrical properties result from the polarity of alternating CH_2 and CF_2 groups on the polymer chain. It withstands exposure to harsh thermal, chemical, or ultraviolet conditions. PES, due to its non-crystalline nature, is attacked by highly polar solvents: such as esters, ketones and trichloroethylene while PVDF is resistant to most chemicals and solvents. PES membranes generally have lower roughness than PVDF membranes which could be an interesting difference with regards to peptide migration or peptide deposition onto the membranes surfaces (Rong G. *et al.* 2005, Zhang Q., *et al.* 2002, & Huyen T., *et al.*2006).

3. Materials and Methods

3.1. Materials

BiPro protein was purchased from Davisco Foods International Inc. (Minnesota, USA). Bovine pancreatic trypsin was purchased from Sigma– Aldrich (St. Louis, MO, USA). PVDF and PES ultrafiltration (both with MWCO 50 kDa) membranes were purchased from Synder Filtration (California, USA). Ion exchange membranes were supplied by Eurodia Industries (PERTUIS, France). HCl and NaOH solutions were obtained from Fisher Scientific (Montreal, QC, Canada). NaCl and KCl were purchased from ACP Inc. (Montréal, QC, Canada).

3.2. Configuration of EDUF

The electrodialysis cell used for our experiment was MP type cell (100 cm^2 of effective surface area) manufactured by ElectroCell Systems AB Company (Täby, Sweden). The configuration was the same as the one used by Doyen *et al.* (2013) (Fig 2.1). Briefly, EDUF configuration consisted of one Neosepta CMX-SB cationic membrane (Tokoyuma Soda Ltd, Tokyo Japon), one Neosepta AMX-SB anionic membrane (Tokoyuma Soda Ltd, Tokyo, Japan) and two polyether sulfone or two polyvinylidiene floride UFMs with a molecular weight cut-off (MWCO) of 50 kDa (Synder Filtration Inc. Vacaville, CA USA)

The configuration consisted of 4 compartments. Two of them, containing 2 L of aqueous KCl (2 g/L) were used for the recovery of recovery of peptides (anionic (A^-_{RC}) and cationic (C^+_{RC}) peptide recovery compartments): they were located near the anode and the cathode respectively. The third compartment contained the electrode NaCl rinsing solution (3 L, 20 g/L), and, the last compartment contained the feed solution (BiPro, 2 L) at 12.5 g/L. The solutions were circulated using four centrifugal pumps and the flow rates were controlled using flowmeters (figure 2.1). Permeate and feed solution flow rates were 1.5 L/min while the flow rate of the electrode solution was 2 L/min (figure 2.1).

3.3. Hydrolysis and Separation Procedures

BiPro solution was prepared by a overnight hydration of 25g of BiPro in 2L of distilled water (1.25% w/v) in a cold room of 4^0C.Trypsic hydrolysis of BiPro carried-out in two setups: one in a beaker (ex-situ) with continuous stirring of the BiPro solution and the other in the EDUF system (in-situ) simultaneously with separation after the pH was adjusted to 7.8. Enzymatic hydrolysis was started by the addition of 10 mL trypsin solution (125 mg/L of trypsin (w/v)). In both in-situ and ex-situ digestions the hydrolysis was performed for 120 minutes after which the enzymatic reaction was stopped by raising the temperature of the solution to 80^0C for 30 minutes.

A constant electric field of 8.22 V/cm was applied between EDUF electrodes. The hydrolysis and fractionation procedures were performed during 120 min. The system was started initially at room temperature and the EDUF parameters are recorded every 15 min during the 120 min experiment. During EDUF, the reaction was maintained at pH 7.8, corresponding to the optimum pH value of trypsin, with 0.5 M NaOH using a pH meter from Thermo Scientific Orion 9206BN probe (VWR International Inc., Mississauga, Ontario, Canada). The pH of recovery compartments was also maintained at 7.8 by a continuous addition of NaOH and HCl by using the same type of pH meter. 10mL of samples from the hydrolysate and each recovery compartment were collected before applying voltage and every 30 min during the treatment from the A^-_{RC}, C^+_{RC} and the feed/hydrolysate compartments. Samples are heated to 80^0C to stop the action of the enzyme. Following each EDUF treatment, the final volumes of A^-_{RC}, C^+_{RC} and the feed/hydrolysate compartments were recovered and freeze dried for storage. Finally a clean-in-place procedure for the EDUF cell was performed after every repeat of EDUF to ensure the recovery of the UFMs and IEMs performances.

Our experiment was performed in two parts. Part one involved EDUF experiments, in an in-situ and ex-situ setup, with both PES and PVDF membranes. In the second part EDUF, in both ex-situ and in-situ setups, was done only with only PVDF membranes and the recovered peptides were analyzed by HPLC-MS in only the second part.

3.4. Analysis

3.4.1. Membrane thickness

Membrane thickness was measured using a Mitutoyo Corp. digimatic indicator (model ID-110 ME, Japan) and a digimatic mini-processor (model DP-1HS, Japan) specially designed for plastic film thickness measurement. The resolution was of 1 μm and the range of 10 mm.

3.4.2. Membrane Conductivity

The membrane electrical conductivity was measured according to the method of Bazinet and Araya-Farias M, using a specially designed clip from the Laboratoire des Matériaux Echangeurs d'Ions (Université Paris XII, Creteil, Val de Marne, France).

3.4.3. Zeta potential measurements

A SurPASS electrokinetic analyzer (Anton Paar, Graz, Austria) equipped with a clamping cell was used to measure the zeta potential of UFMs. The determination of zeta potential with the SurPASS electrokinetic analyzer is based on the streaming current or streaming potential measurement created by the circulation of the electrolyte through a capillary system. The streaming current was measured in 1 mM KCl solution in a pH range of 2.5-11. The streaming channel of well-defined dimensions (25 mm in length and 5 mm in width) was formed by two identical flat membranes mounted opposite of each other and separated by one spacer. The streaming current was measured alternatively in the two flow directions by pressure ramps in the range 0 to 300 mbar. Two cycles of pressure ramps in each direction were conducted and measured average zeta potential values were computed using the Fairbrother-Mastin model.

3.4.4.Measurement of Solution Conductivities

Conductivities of A^-_{RC}, C^+_{RC} and feed compartments were measured every 15 min during the 120 min of EDUF with a YSI conductivity meter (model 3100) equipped with a YSI immersion probe (model 3252, cell constant K = 1 cm^{-1}, yellow Springs Instrument Co., Yellow springs, OH, USA). The conductivities were measured in order to evaluate the mineralization or demineralization of the solutions during the process.

3.4.5. Total Peptide Determination in Different Compartments

Total peptide migrations to the A^-_{RC}, C^+_{RC} and feed compartments were determined from samples withdrawn every 30 min over a period of 120 min using the BCA protein assay (Pierce, Rockford, IL, USA). The microplate was first incubated 37^0C and then cooled to room temperature and the absorbance was read at 562 nm on a microplate reader (THERMOmax, Molecular devices, Sunnyvale, CA). Concentration was determined with a standard curve in a range of 5–2000 µg/mL of bovine serum albumin (BSA).

3.4.6. Protein and Peptide Profile with HPLC

The peptide composition of the A^-_{RC}, C^+_{RC} and hydrolysate solutions was determined by RP-HPLC according to the method of Firdaous *et al.* (2010) adapted to the specific conditions of the feed and peptides generated during hydrolysis. The system used was an Agilent 1100 series. Peptides were analyzed with a Luna 5 lm C18 column (2 i.d. x 250 mm, Phenomenex, Torrance, CA, USA). Solvent A, TFA 0.11% (v/v) in water, and solvent B, acetonitrile/water/ trifluoroacetic acid (TFA) (90%/10%/0.1% v/v), were used for elution at a flow rate of 10 µL/min. A linear gradient of solvent B, from 3% to 60% in 85 min, was used. The detection

wavelength was 214 nm which is typically used to monitor peptide bonds (Firdaous *et al.*, 2009, and Stachelhaus, T., *et al.* 1998).

3.4.7. Peptide Molecular Weight Determination

Protein and peptide molecular weights were determined by using MS. MS analysis were performed with a scan range of 300 - 2200 m/z in positive polarity, with an ESI ion source type, at a dry temperature of 350°C, a nebulizer at 30.00 psi and dry gas of 8.00 L/min.
The molecular weight (MW) of proteins and peptides in recovered samples were determined by mass spectrometry (LC-MS) analyses according using ion trap method. The system used was an Agilent 1100 series (Agilent Technologies, Palo Alto, CA, USA). Peptides were analyzed with the same method and the same column used for RP-HPLC analyses. To reduce the effect of TFA, mass-spectrometry was performed after infusing (10 µL/min) a mixture of 50% propionic acid and 50% isopropanol to the existing flow before the MS interface. Signals were recorded in positive mode using a 90-V fragmentation with a scan range of 300–3000 m/z (Firdaous *et al*., 2009).

3.5. Statistical Analysis

The changes in thickness, conductivity, zeta potential, peptide concentration and system parameters were subjected to repeated measures analysis of variance ($P < 0.05$ as probability level for acceptance) using sigma plot integrated software (Systat Software, Inc San Jose, CA, USA).

4. Result and Discussion

4.1. Studies with PES and PVDF Membranes

4.1.1. Membrane characterization

A. Membrane Thickness

Membrane thickness and conductivity were determined for every membrane used before and after EDUF experiments. Measurement of membrane thickness can be a rapid method to follow effects of electrodialysis (ED) or EDUF on membranes (Casademont C., *et al.,* 2010). The mean values together with the standard deviations of quadruplicate measurements are presented in Table 1.

ANOVA showed that membrane material, PES and PVDF, had a significant effect on membrane thickness ($P<0.001$) with averages for all conditions being of 0.194 ± 0.007 mm and 0.234 ± 0.016 mm, respectively (table 1). Especially for PES membrane, ANOVA showed that its thickness varied significantly before and after EDUF ($P<0.002$), with UFM number (UFM-I or UFM-II, $P<0.001$) and with ex-situ vs in-situ experiments ($P<0.001$). Moreover double interaction were also detected between ex-situ/in-situ experiments and before/after EDUF ($P<0.022$), ex-situ/in-situ experiments and UFM number ($P<0.011$) and before/after EDUF and UFM number ($P<0.022$). No triple interactions were detected. For PVDF membrane thickness, ANOVA indicated a significant difference only for ex-situ vs in-situ ($P<0.001$) and for UFM number ($P<0.001$). For IEMs, their thicknesses varied significantly with IEM type (AEM vs CEM, $P<0.001$) and UFM type with which it was used (PES vs PVDF, $P<0.001$). The control AEM and CEM had mean thicknesses of 0.140 ± 0.003 mm and 0.170 ± 0.003 mm, respectively.

PES UFMs showed significant change in thickness while PVDF indicated no significant change in thickness which may suggest a slight difference in susceptibility to surface deposition of foulants. Marginal differences in thickness of UFM-I and UFM-II of same membrane types could be due to differences in storage and position of folding during the rolling of membrane for shipment and storage. The other types of membranes; AEM and CEM showed no a significant change in thickness as a result of EDUF. Although change in thickness of AEM after use in ED has been reported before (Casademont C., *et al.,* 2010), we observed no significant difference in thickness of AEM after being used in our EDUF experiments. Due to the nature of the driving force involved, which is applied electric field, the possibility of having uncharged peptides depositing on the surface of UFMs is reduced compared to pressure driven processes (Poulin J., *et al.,* 2006).

Table 1: Thickness (mm) and conductivity (mS/cm) of UFMs and IEMs used in EDUF experiments

			Thickness [mm]		Conductivity [mS/cm]	
UFM Material	**Digestion Strategy**	**Membrane Type**	**Before EDUF**	**After EDUF**	**Before EDUF**	**After EDUF**
PES	Ex-situ	UFM-I	0.193 ± 0.003^{a}	0.189 ± 0.002^{b}	6.48 ± 0.24^{a}	6.04 ± 0.19^{b}
		UFM-II	0.200 ± 0.003^{c}	0.206 ± 0.005^{d}	6.68 ± 0.16^{c}	6.31 ± 0.11^{d}
		AEM	0.142 ± 0.002^{a}	0.140 ± 0.002^{a}	9.09 ± 0.18^{a}	7.55 ± 0.19^{b}
		CEM	0.176 ± 0.002^{c}	0.176 ± 0.001^{c}	6.26 ± 0.07^{c}	3.28 ± 0.31^{d}
	In-situ	UFM-I	0.185 ± 0.003^{a}	0.191 ± 0.001^{b}	5.94 ± 0.19^{a}	5.85 ± 0.54^{b}
		UFM-II	0.190 ± 0.003^{c}	$0.198 \pm 0 .003^{d}$	4.98 ± 0.31^{c}	4.00 ± 0.38^{d}
		AEM	0.140 ± 0.003^{a}	0.144 ± 0.002^{a}	9.13 ± 0.74^{a}	6.61 ± 0.24^{b}
		CEM	0.175 ± 0.002^{c}	0.173 ± 0.002^{c}	10.76 ± 0.09^{a}	10.3 ± 0.31^{c}
PVDF	Ex-situ	UFM-I	0.238 ± 0.005^{a}	0.242 ± 0.003^{a}	3.13 ± 0.12^{a}	1.59 ± 0.10^{b}
		UFM-II	0.212 ± 0.001^{c}	0.215 ± 0.002^{c}	2.73 ± 0.32^{c}	1.58 ± 0.29^{d}
		AEM	0.139 ± 0.001^{a}	0.139 ± 0.001^{a}	9.12 ± 0.09^{a}	7.6 ± 0.09^{b}
		CEM	0.171 ± 0.001^{c}	0.171 ± 0.002^{c}	11.19 ± 0.16^{a}	8.64 ± 0.12^{c}
	In-situ	UFM-I	0.255 ± 0.003^{a}	0.256 ± 0.002^{a}	5.23 ± 0.04^{a}	5.52 ± 0.20^{b}
		UFM-II	0.227 ± 0.001^{c}	0.225 ± 0.005^{c}	4.27 ± 0.18^{c}	4.64 ± 0.22^{d}
		AEM	0.139 ± 0.002^{a}	0.143 ± 0.003^{a}	8.56 ± 1.14^{a}	6.64 ± 0.13^{b}
		CEM	0.172 ± 0.003^{c}	0.173 ± 0.002^{c}	10.66 ± 0.16^{c}	7.99 ± 0.15^{d}

Different letters, in a row or a column, indicate statistically significant difference (P=0.005) for the parameter/membrane of interest.

B. Membrane Conductivity

Statistical analysis of variance (ANOVA) indicated that membrane material (PES/PVDF) had a significant effect on the conductivity of UFMs ($P<0.001$) with overall mean values of 5.83 ± 0.88 mS/cm and 3.59 ± 1.49 mS/cm for PES and PVDF, respectively. ANOVA also indicated that there is a significant difference in PES membrane conductivity used for ex-situ vs in-situ experiments ($P<0.001$), before and after EDUF ($P<0.001$) and UFM number ($P<0.001$). Significant interaction was also seen between UFM number and before and after EDUF ($P<0.001$). Within both UFM-I and UFM-II a significant difference in conductivity was observed for ex-situ vs in-situ experiments ($P<0.005$, for both). Within PES in-situ experiments significant differences in conductivity are indicated for UFM number ($P<0.005$) (Table 1).

ANOVA also indicated a significant difference in conductivity of PVDF membranes used in ex-situ and in-situ experiments ($P<0.001$), before and after EDUF ($P<0.001$) and UFM number ($P<0.001$). Within both PVDF UFM-I and UFM-II significant difference in conductivity was observed for ex-situ vs in-situ experiments ($P<0.001$, for both) (Table 1). Within PVDF in-situ experiments significant differences in conductivity are observed for UFM number ($P<0.001$) and significant differences were also seen within before EDUF (3.13 ± 0.12 mS/cm and 5.23 ± 0.04 mS/cm, for UFM-I ex-situ and in-situ, respectively, $P<0.001$) and within after EDUF ($P<0.001$) for ex-situ vs in-situ experiments (Table 1).

Amongst the UFMs, PES UFM-II used in ex-situ experiment exhibited the highest conductivity at 6.68 ± 0.36 mS/cm while PVDF UFM-II showed the lowest conductivity at 2.73 ± 0.32 mS/cm. PVDF membranes used as both UFM-I and UFM-II particularly in the ex-situ experiment had significantly ($P<0.0036$) lower conductivities (3.13 ± 0.12 mS/cm and 2.73 ± 0.32 mS/cm, respectively) compared to other PVDF UFM-I and UFM-II membranes used in the in-situ experiment (5.23 ± 0.05 mS/cm and 4.27 ± 0.19 mS/cm, respectively).

Electrical conductivities of UFMs are not provided by manufacturers as the main market and researchers that use/study UFMs use them in pressure driven processes for which MWCO are more important than electrical conductivity. However Donose *et al.,* (2011) reported that conductivity can indeed be used to check the integrity of RO and NF membranes as a surrogate measurement for rejection of ions by the membranes. In the same manner the conductivity of UFMs can be used to check for relation to peptide migration across membranes in EDUF and check for structural integrity of membranes. Lower conductivities for the original membranes observed for UFM-I and UFM-II used in PVDF ex-situ experiment can, therefore, suggest certain structural anomalies that can lead to vulnerability to fouling which is indicated by the significant decrease, almost by half, of conductivity in these membranes after use in EDUF (table 1). In fact these aberrations were sometimes apparent in the different physical appearance of membranes obtained from the same roll/sheet and this could be the reason why membranes had such a significantly different conductivity before EDUF.

In a study reported by Doyen *et al.,* (2011), the conductivity of PES membrane changed significantly after use in EDUF while no change was detected for cellulose acetate (CA) membrane used in the same setup of EDUF. In our case both PES and PVDF membranes exhibited significant decrease in conductivity probably because of their hydrophobic nature as opposed to the hydrophilic CA membrane. The hydrophobic nature of the membranes can contribute to fouling because most peptide-membrane interactions that cause fouling has been reported to be of hydrophobic nature (Groleau *et al., 2003)*.

For IEMs ANOVA showed that IEM material had no significant effect on the conductivity of IEMs while there was a significant decrease in IEM conductivity after use in EDUF ($P<0.001$). Marginal differences in IEM conductivity were detected for the two types of (PES and PVDF) UFMs the IEMs were used with ($P=0.033$). Double interactions were detected between UFM type and IEM type ($P=0.012$). ANOVA also showed a significant difference in IEM conductivity used in ex-situ and in-situ experiments ($P=0.012$) (Table 1).

The least IEM conductivity before EDUF was recorded for CEM membrane used with PES ex-situ experiment (6.26 ± 0.07 mS/cm) while the largest was recorded for CEM membrane used in PVDF ex-situ experiment (11.19 ± 0.16 mS/cm). For AEM the largest conductivity was recorded for the one used in PES in-situ experiment (9.13 ± 0.74 mS/cm) while the smallest was recorded for the one used in PVDF in-situ (8.56 ± 1.14 mS/cm). A drastic decrease in conductivity was observed for CEM membrane used with PES ex-situ experiment from 6.26 ± 0.07 mS/cm to 3.28 ± 0.31 mS/cm, which accounts for a 47.6 % decrease; the largest percentage decrease observed for any IEM we used. This is in line with the observation that UFMs that had the least conductivity before EDUF showed the most drastic reduction in conductivity after use in EDUF suggesting that, in both UFMs and IEMs, lower membrane conductivities are indication of membrane structural aberrations, possible attained during shipment and/or storage,that made the membrane more susceptible to fouling.

IEM fouling is one of the major problems in milk or complex food systems electrodialysis (Casademont C. *et al.,* 2006). Fouling in IEMs during ED and its follow up by decreasing conductivity (increasing resistance) has been reported before (Lindstrand V. *et al*. 2007 & Lindstrand V. *et al*., 2000). In line with reports made by Lindstrand *et al.,* (2007) both CEM and AEMs had significantly ($P<0.001$) reduced conductivity after being used in EDUF which is an indication of fouling which is caused by the deposition of peptides or amino acids (organic materials) resulting from the digestion of the BiPro or by deposition of inorganic minerals (Casademont C., *et al.,* 2010) because BiPro also contains 0.6% minerals as indicated by the manufacturer (DAVISCO).

C. UFM Zeta Potential

Figure 4.1 below displays the evolution of zeta potential of both PES and PVDF membranes as a function of pH. As can be seen from the figure both UFMs membranes showed a similar trend: zeta potential was higher in the pH range 2-3: the highest zeta potential was recorded at pH 2.49 for PES (0.25 ± 2.03 mV) and at pH 2.84 for PVDF membrane (9.20 ± 2.93 mV). After pH 3 the zeta potential dropped rapidly to negative values till pH6, then it remained constant between pH 6 to 9. Past pH 9 the zeta potential again dropped rapidly for both PES and PVDF

membranes to attain a close value of -29.12 ± 1.36 mV and -26.29 ± 2.44 mV, respectively at pH 11.15.

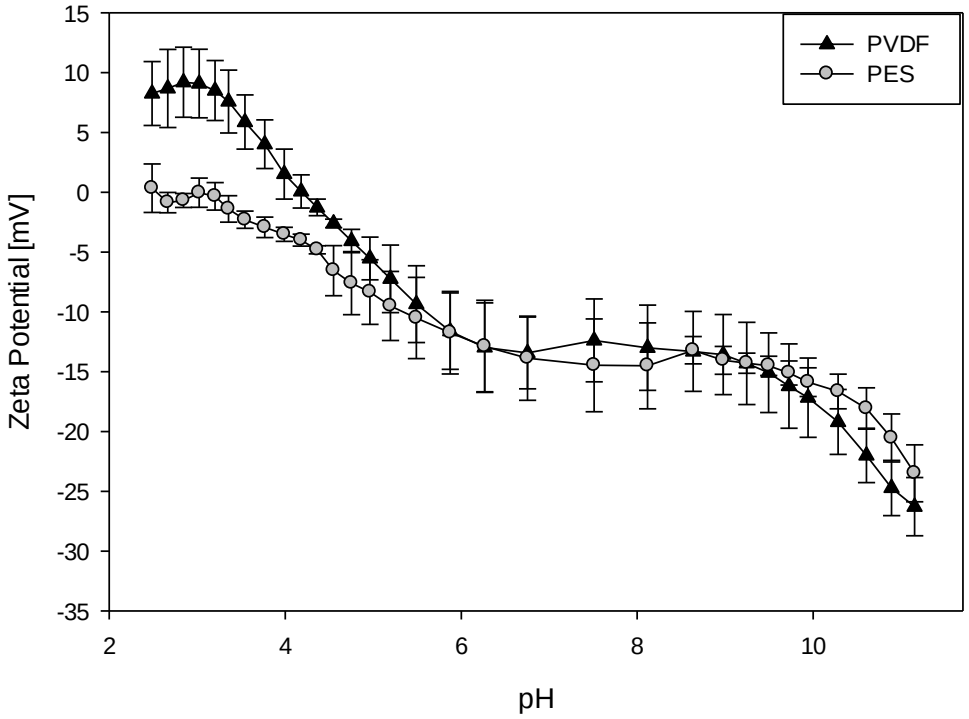

Figure 4.1: Evolution of Zeta potentials of PES and PVDF UFMs as a function of pH

However there are notable differences, for almost the entire range of pH 2.5 to 11, PES membrane showed a negative zeta potential except at very acidic pH (pH 2.5–3) were the membrane surface potential was close to zero. For the pH range 2.5 to 4 PVDF membrane showed positive zeta potential; for instance at pH 2.5, PVDF zeta potential was recorded at 8.26 ± 2.67 mV as opposed to 0.35 ± 0.35 mV recorded at the same pH for PES. The other notable difference was the point of zero charge; PVDF attained this point at pH 4.17 while PES attained it at pH 2.96. Interestingly though near the pH of EDUF operation, which is 7.8, PES and PVDF show close zeta potential measurements; -13.20 ± 1.14 mV and -12.37 ± 3.46 mV, respectively. Two iso-potential points, the pH where both membranes attain the same zeta potential, were identified from the pH titration of the two UFMs (figure 4.1), one at pH 5.87 and the second at pH 8.63.

Similar trend of zeta potential evolution with pH for PES and PVDF membranes have been reported before (Kim K., *et al.,* 1996). The observation that the PVDF membranes showing a positive charge a lower pHs and negative charge at higher pHs could be due to adsorption and

dissociation of ions like H^+, K^+, Cl^- and OH^- from the solution onto the membrane surface (Kim K., *et al.,* 1996). Surface dissociable groups could explain the negative zeta potential observed for PES membranes as the membrane has previously been reported to behave as a weakly acidic material (Lara R., and Benavente J., 2009). Due to the close zeta potentials exhibited by the PES and PVDF membranes at the working pH (7.8) (-13.20 ± 1.14 for and -12.37 ± 3.46, respectively) we expect no significant difference in terms of peptide migration with respect to the membranes on the basis of this little difference in zeta potential.

4.1.2. Electrodialysis with Ultrafiltration Membranes (EDUF) Parameters

4.1.2.1. Evolution of System Temperature

Figure 4.2 depicts the variation of temperature in the feed compartment as a function of time. No statistically significant difference evolution of temperature with time was observed for the different membranes used but temperature significantly changed with time ($P<0.001$) during the first half of EDUF. Statistically significant difference in initial temperature was observed for PES ex-situ experiments (29.97 ± 0.60 ^{0}C) and PES in-situ experiments (at 32.87 ± 1.43 ^{0}C) ($P<0.001$). System temperature generally increased from initial values to 35-36 ^{0}C within the first 60 minutes of EDUF. As can be seen from figure 4.1 and as tested by ANOVA significantly different evolutions of temperature ($P<0.001$) with time is observed for ex-situ vs in-situ experiments, regardless of the membrane type used; the average temperature for all in-situ experiments was 35.55 ± 1.78 ^{0}C and for all ex-situ experiments was 33.52 ± 2.03^{0}C.

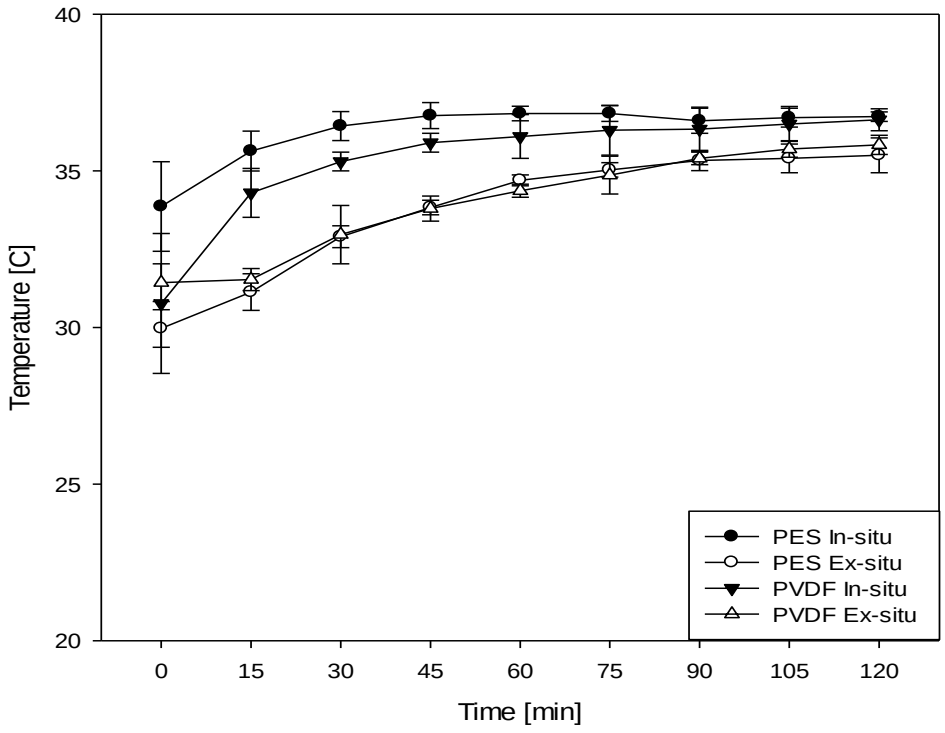

Figure 4.2: Evolution of feed temperature as a function of time during 120 minutes of EDUF

The increase in temperature can be explained by joule heating, where the passage of current through a system heats the system (Firdaous *et al* ., 2009), and also the effect of pumping used for re-circulating solutions in the four compartments of feed, A^-_{RC} , C^+_{RC} and the electrolyte solution (near the cathode and near the anode) compartments. Such phenomena are well documented in EDUF systems and previously reported elsewhere (Firdaous *et al*., 2009). The slight variation in temperature between in-situ and ex-situ experiments could be due to minor variations in room temperatures that are less likely to affect EDUF parameters. Here it is important to note that the temperature in the recirculation system stabilizes near 36^0C and never over passes 37^0C making the use of external heating system to maintain the feed temperature at optimum for protein digestion by trypsin (37^0C) a non-necessity.

4.1.2.2. Evolution of System Resistance

Figure 4.3 below displays the change of system resistance as a function of time. Statistical analysis using ANOVA showed system resistance evolved significantly differently between the two UFMs used ($P<0.001$) and a significant difference in system resistance evolution was also detected in ex-situ vs in-situ within PES ($P<0.001$) and within PVDF ($P<0.001$) membranes. A

significant difference in system resistance is also seen for the different UFMs within in-situ ($P<0.001$) and also within ex-situ ($P<0.001$) experiments.

In all the experiments a similar trend of evolution of system resistance is observed; resistance decreased during the first 45 minutes of EDUF and it slightly increased during the next 75 minutes. For all the EDUF experiments maximum system resistance was attained at t=120 minutes, this maximum resistance was the highest for PVDF ex-situ at a value of 55.49 ± 6.42 Ω and the lowest for PVDF in-situ at a value of 44.55 ± 5.14 Ω. As can be seen from figure 4.3, experiments which started with higher resistance maintained a higher resistance throughout the EDUF experiment till the end while those experiments that started with a lower resistance maintained it till the end compared to the other experiments.

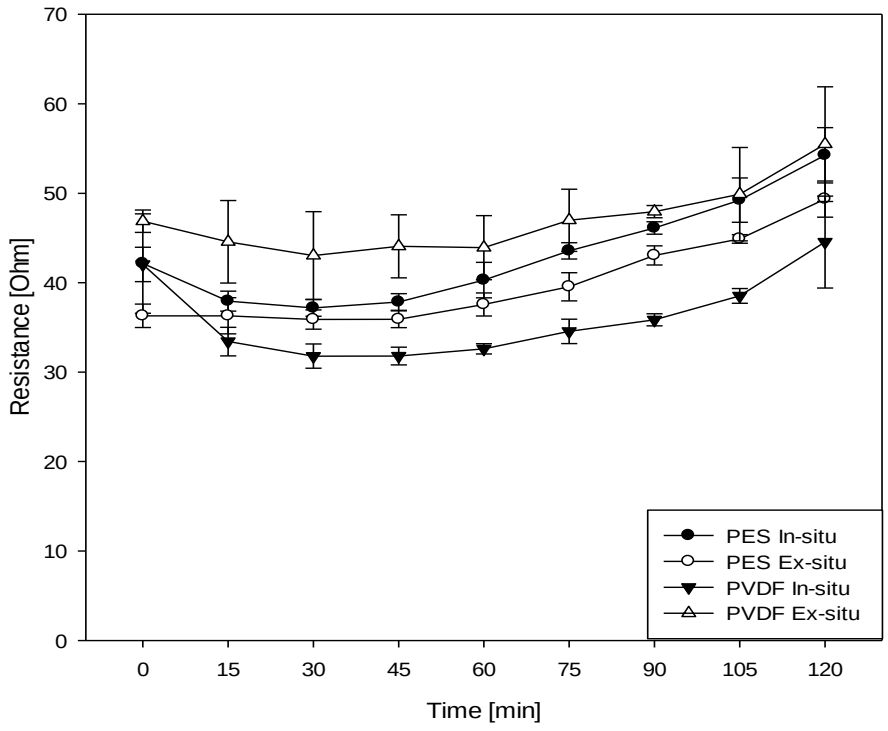

Figure 4.3: Evolution of system resistance as a function of time during 120 minutes EDUF

During initial times of EDUF, the rise in temperature reported in the above section (4.1.2.1) is accompanied by a corresponding decrease in system resistance. This suggests an expected inverse relationship between temperature and system resistance (Yang H., *et al.*, 2008) at initial times of EDUF. As we cross the initial 45-60 minutes of EDUF we observed a constant system temperature (figure 4.1) but a slight, and continuous, increase in resistance was also observed (figure 4.3). This could be attributed to the increasingly lower concentration of K^+ and Cl^- ions in the A^-_{RC} and C^+_{RC}, with time, due to their net migration into the feed and electrolyte

compartments giving rise to a lower current for the same applied voltage, hence a higher resistance.

On a closer look the variation of the system resistance for the different experimental condition it is observed that PVDF ex-situ experiments exhibited a higher resistance than the rest of the other three experiments. This could be attributed to the significantly lower conductivity exhibited by the PVDF membranes used as UFM-I and UFM-II in the PVDF ex-situ experiment. In this experiment the UFM-I and UFM-II had conductivities of 2.7 ± 0.32 mS/cm and 3.13 mS/cm ± 0.12 mS/cm, respectively, compared to the other membranes which have conductivities close to 5 mS/cm. For instance UFM-I and UFM-II in PVDF in-situ experiments had conductivities of 5.23 ± 0.05 mS/cm and 4.67 ± 0.19 mS/cm, respectively and showed the lowest system resistance when used in EDUF. Experiments with PES membranes showed no significant difference in system resistance evolution for ex-situ/in-situ but when compared to their PVDF counterparts PES experiments exhibited a slightly higher system resistance compared to PVDF membranes with relatively close conductivity values (Example, PES in-situ experiments, UFM-I and UFM-II had conductivities of at 5.94 ± 0.19 mS/cm and 4.98 ± 0.31 mS/cm but had a system resistance slightly higher than PVDF in-situ experiment mentioned above). This observation suggests that, when the conductivities are close to each other, PVDF membranes impart lesser system resistance than PES.

4.1.2.3. Evolution of Conductivity in the Different Compartments

The evolution of conductivity as a function of time in the A^-_{RC} for the in-situ and ex-situ experiments with both UFMs is presented in figure 4.4. In all the experiments a linear decrease of conductivity is observed regardless of membrane type and digestion strategy used. ANOVA showed no significant difference in A^-_{RC} conductivity for membrane type or strategy of digestion but there was a significant effect of time ($P<0.001$) on A^-_{RC} conductivity. Average extent of demineralization in A^-_{RC} for all the EDUF experiments was calculated to be 67.46 ± 2.00 % and there was no statistically significant difference in extent of demineralization in the A^-_{RC} for the different UFMs used.

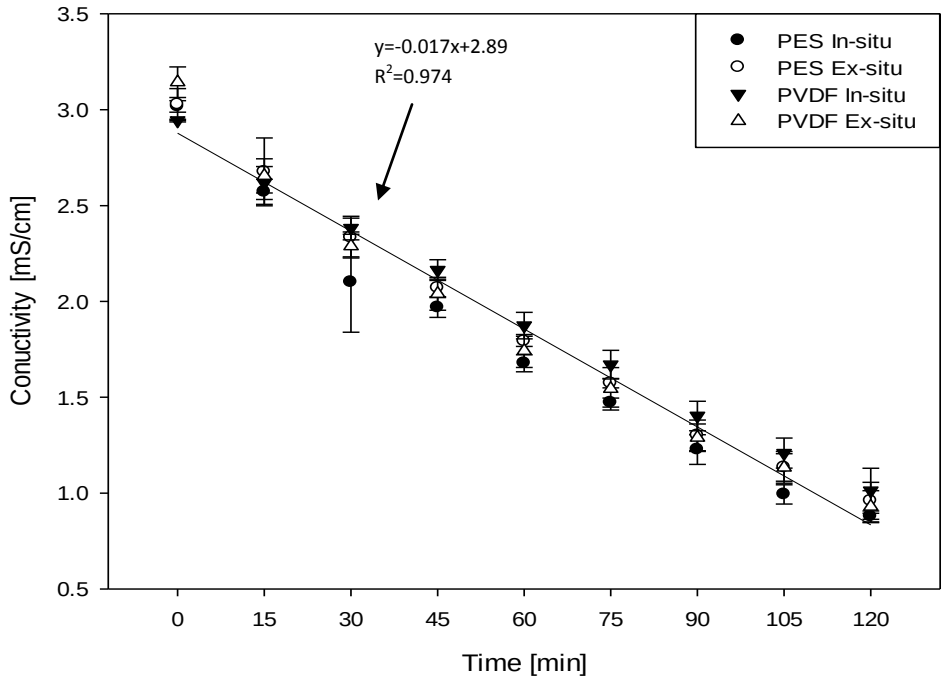

Figure 4.4: Evolution of conductivity in the anionic peptide recovery compartment (A^-_{RC}) for in-situ and ex-situ experiments with PES and PVDF membranes

A similar trend of linearly decreasing conductivity with time is also observed for the C^+_{RC} for both UFMs and for both ways of digestion as indicated by the global regression lines in figure 4.5. Marginally significant difference in C^+_{RC} conductivity evolution with time was observed for PES vs PVDF membrane types ($P<0.04$) as tested by ANOVA which also indicated a significant effect of time ($P<0.001$) on C^+_{RC} conductivity. For the extent of demineralization in the C^+_{RC} significant difference ($P<0.001$) was observed for PES and PVDF membranes with mean values of (across in-situ and ex-situ experiments) 39.43 ± 6.22 % and 52.22 ± 3.43 %, respectively. Average extent of demineralization in C^+_{RC} for all the EDUF experiments was calculated to be 45.83 ± 8.16 %. This is confirmed by slope of the global regression line for the decrease of conductivity in C^+_{RC}, with PES and PVDF membranes, which are -0.0104 mS cm^{-1} min^{-1} and -0.0134 mS cm^{-1} min^{-1}, respectively. Hence, there was a slight difference on the rate at which conductivities changed for the two membranes. This is also related to the system resistance evolution reported in the previous section where system resistance were generally lower for PVDF membranes than PES when the two types of membranes have closer values of conductivity.

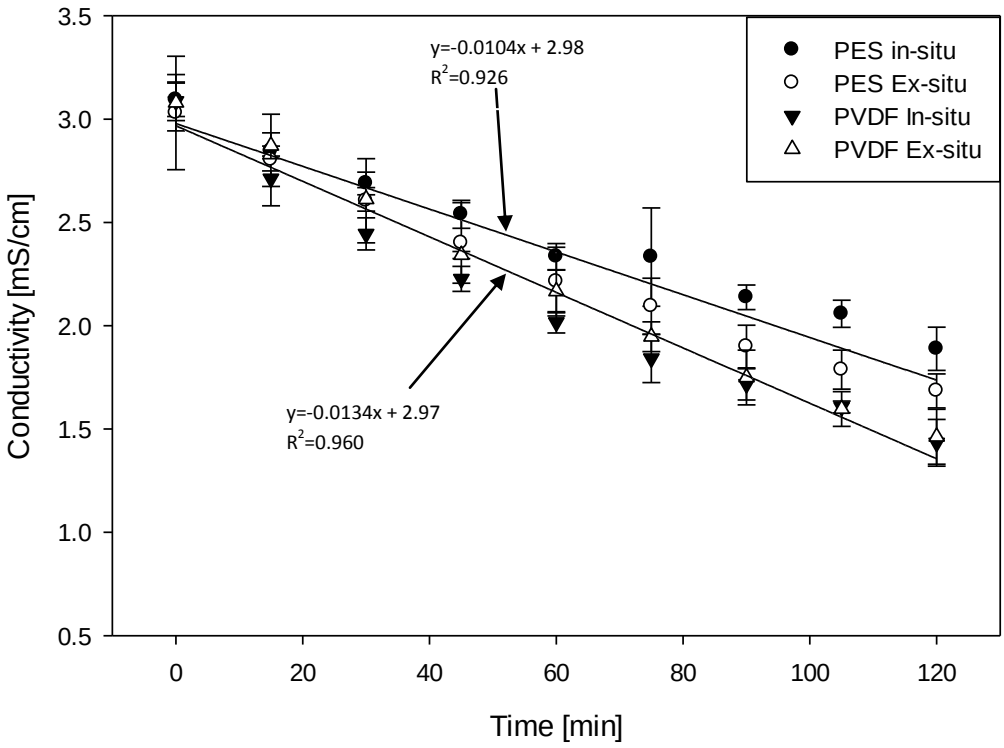

Figure 4.5: Evolution of conductivity in the cationic peptide recovery compartment (C^{+}_{RC}) for in-situ and ex-situ experiments with PES and PVDF membranes

As the conductivity in the two peptide recirculation compartments were decreasing, the conductivity in the feed recirculation compartment was increasing as indicated in figure 4.6. ANOVA indicated no significant difference in feed compartment conductivity for PES/PVDF membranes used or for the digestion strategies employed but there was a significant effect of EDUF duration on the compartment conductivity ($P<0.001$). The average initial conductivity in the feed compartment for all the experiments was 0.656 ± 0.144 mS/cm and it reached 1.577 ± 0.244 mS/cm at the end of EDUF (2hrs).

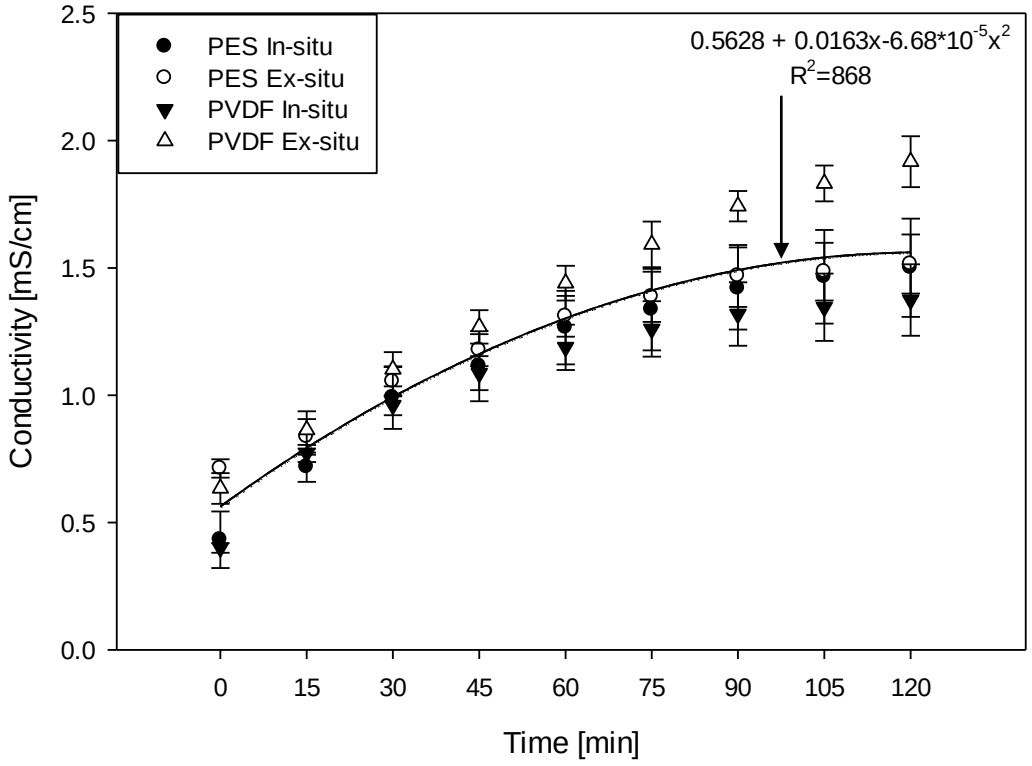

Figure 4.6: Evolution of conductivity in the feed recirculation compartment for PES in-situ, PES ex-situ, PVDF in-situ and PVDF ex-situ EDUF experiments

The average extent of mineralization in the feed recirculation compartment across all experiments was calculated to be 63.8 ± 8.20%. ANOVA showed that mineralization (starting from initial time to final time) in this compartment had a statistically significant difference for in-situ vs ex-situ experiments ($P<0.001$) at mean values of 68.83 ± 3.6 % and 58.9 ± 8.23 %, respectively.

The decrease in conductivity in the A^-_{RC} is due to the migration of Cl^- ions towards the anode passing through the AEM to end up in the electrolyte recirculation compartment near anode. At the same time the K^+ ions migrate towards the cathode passing through the feed recirculation on their way to electrolyte solution near the cathode (Figure 2.1). The same phenomenon can explain the decrease of conductivity in the C^+_{RC}, the K^+ ions migrate to the electrolyte solution near the cathode passing through the CEM while the Cl^- ions migrate to the electrolyte recirculation near the cathode passing through the feed compartment. The increase in conductivity in the hydrolysate compartment is due to the transfer of K^+ ions migrating across UFM-I from the A^-_{RC} and the transfer Cl^- ions migrate across UFM-II from C^+_{RC} (Doyen, *et al.*, 2013). The generation of anionic and cationic peptides from tryptic digestion of BiPro proteins also contributed to the increase in conductivity with time in the hydrolysate compartment as seen during the ex-situ (beaker) hydrolysis. Similar trends of linearly decreasing conductivities

in A^-_{RC} and C^+_{RC} and increasing conductivities in the feed recirculation compartment were reported before (Roblet C., *et al.*, 2013). It is important to note that conductivities are directly affected by the NaOH and HCl added in all compartment to keep the pH of all the compartments at 7.8 to avoid the possibility of having peptides assume different charges in different compartments if the pH had changed from one compartment to the other.

4.1.3. Total Peptide Migration

Figure 4.7 below shows the total peptide that migrated into the A^-_{RC} with time for different digestion strategy used with both PES and PVDF UFMs. ANOVA indicated total peptide migration in the A^-_{RC} is significantly affected by membrane type ($P<0.001$), strategy of digestion ($P<0.001$) and time ($P<0.001$). Double interactions were detected between membrane type and ex-situ/in-situ digestion ($P<0.001$), membrane and time ($P<0.001$), ex-situ/in-situ with time ($P<0.001$). Triple interactions were recorded between membrane type, ex-situ/in-situ digestion and time ($P<0.003$).

All experiments showed a similar trend of roughly linear increase, though at different rates, of total peptide migration to the A^-_{RC} as a function of time. The rates of migration began to differ at initial minutes except for PES in-situ and PVDF ex-situ experiments which indicated a similar, and very small, rate of total peptide migration till the end of the first 30 minutes of EDUF treatment.

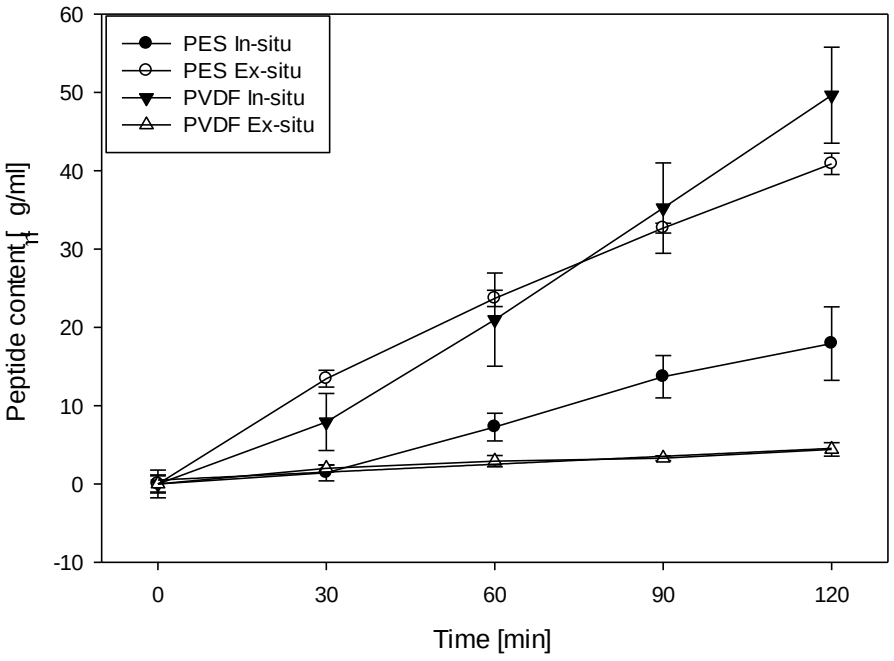

Figure 4.7: Total peptide migration into the A^-_{RC} as a function of time for PES and PVDF membranes with in-situ and ex-situ digestion

Maximum rate of increase was observed for PVDF in-situ experiments with slope of regression line 0.42 µg $ml^{-1}min^{-1}$ (R^2=0.9895) while the minimum was observed for PVDF ex-situ experiment with slope of the regression line 0.0051 µg $ml^{-1}min^{-1}$ (R^2=0.9347).

As it can clearly be seen in figure 4.7 above and as the statistical tests indicated peptide migrations were significantly lower for PVDF ex-situ with maximum value at t=120 minutes equal to 4.41 ± 0.86 µg/ml. This value was much less than the total peptide migration observed for the same membrane but with in-situ digestion, which was 49.65 ± 6.13 µg/ml at t=120 min. For PES membrane ex-situ digestion showed a significantly higher (P<0.001) peptide migration (40.88 ± 1.36 µg/ml) compared to in-situ (22.94 ±4.46 µg/ml) at t=120 min. Hence the nature of digestion is less likely to be the major reason behind the lowest migration observed for PVDF ex-situ experiments because of the reverse effect observed with PES membrane. However on comparing the conductivities of the UFMs reported in table 1 (section 4.1.1.1) and comparing the respective peptide migration depicted on figure 4.7 above, we see a striking effect of UFM electrical conductivity on peptide migration rates across UFMs. A good example is the UFM-I (forming the barrier between hydrolysate and A^-_{RC}) used in PVDF ex-situ experiments which had a conductivity of 3.163 ± 0.12 mS/cm significantly lower (P<0.001) than the UFM-I used in the PVDF in-situ experiment which had 5.23 ± 0.04 mS/cm, that could explain the difference in migration indicated above. The effect of conductivity is also observed within PES membranes, PES UFM-I in the ex-situ experiment had a significantly higher (P<0.001) conductivity (6.43 ± 0.24 mS/cm) compared to the one used in the in-situ experiment (5.94 ± 0.19 mS/cm), explaining the significantly higher (P<0.05) migration for PES ex-situ (40.88 ± 1.36 µg/ml) compared to the in-situ experiment with the same type of UFM (22.94 ± 4.46 µg/ml).

Figure 4.8 presents the total peptide migration to the C^+_{RC} as a function of time. ANOVA indicated that total peptide migration to the C^+_{RC} is significantly affected by membrane type (P<0.001), ex-situ/in-situ digestion (P<0.001), and time (P<0.001). Double interactions were also observed between membrane type and ex-situ/in-situ digestion (P<0.001), membrane type and time (P<0.001), ex-situ/in-situ with time and triple interactions between membrane type, ex-situ/in-situ digestion and time (P<0.001).

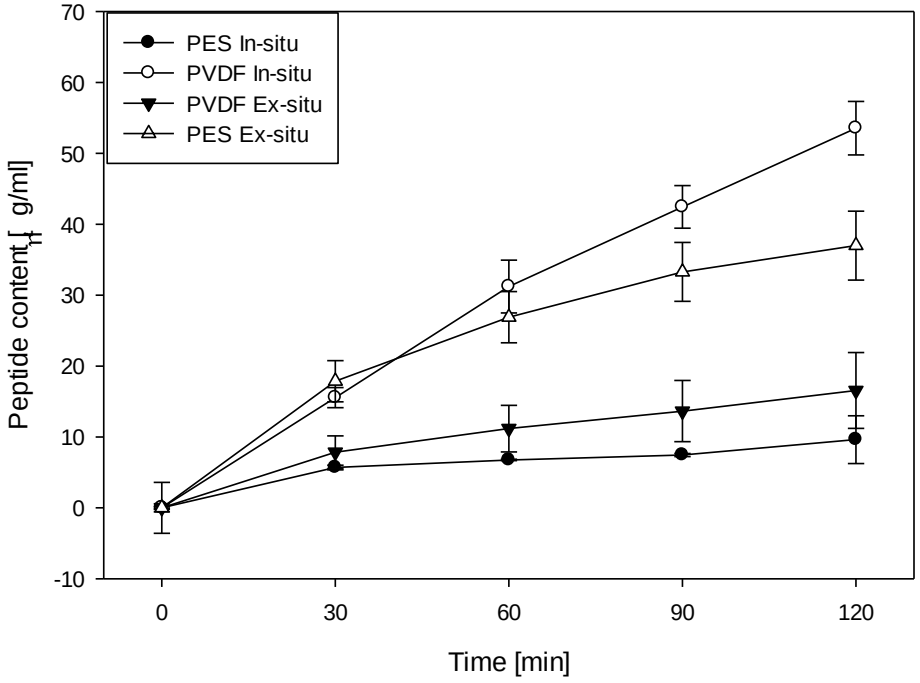

Figure 4.8: Total peptide migration into the C^{+}_{RC} as a function of time for PES and PVDF membranes with in-situ and ex-situ digestion

As in the case of migration into the A^{-}_{RC}, a roughly linear increase of migration as a function of time was observed for C^{+}_{RC}, again at different rates. The maximum rate was observed for PVDF in-situ with a slope of the regression line equal to 0.45 µg $ml^{-1}min^{-1}$ (R^2=0.992) while the lowest was observed for PES in-situ with a slope of regression line equal to 0.07 µg $ml^{-1}min^{-1}$ (R^2= 0.851). Moreover the highest final migration to the C^{+}_{RC} is observed for PVDF in-situ experiment (53.55 ± 3.77 µg/ml) while the lowest was observed for PES in-situ (9.16 ± 3.37 µg/ml). Here again it is important to note the effect of membrane conductivity (reported in table 1 section 4.1.1.1) on the total peptide migration to the C^{+}_{RC}. As migration to the A^{-}_{RC} was affected by the electrical conductivity of UFM-I, the migration to the C^{+}_{RC} is also affected by the conductivity of UFM-II. Hence the UFM-II used for PVDF in-situ (with the highest C^{+}_{RC} migration) had a conductivity of 4.27 + 0.19 mS/cm–the second highest in conductivity amongst all the PVDF membranes used; while UFM-II used in PES in-situ (with the lowest C^{+}_{RC} migration) had a conductivity of 4.98 ± 0.31 mS/cm which is the smallest conductivity amongst all the PES membranes used (Table 1). This suggests that while the membrane type is important, differences in conductivities within the same membrane type are of significant role in determining the total peptide migration to a given compartment.

Moreover it is important to highlight that the EDUF experiment which showed a consistently lower peptide migration, i.e. PVDF ex-situ, also showed a consistently higher system resistance

(Section 4.1.2.2) than the rest of the experiments throughout the 2hrs of EDUF. Similarly the EDUF experiment which showed a consistently higher peptide migration, i.e. PVDF in-situ, also showed a consistently lower system resistance (Section 4.1.2.2) than the rest of the experiments. This observation underlines the importance of UFM conductivity for both system resistance and peptide migration which are inter-related parameters in EDUF.

Linear increase in peptide migration with time during EDUF has been reported by Firdaous *et al.,* (2010) and Poulin *et al.,* (2006) and this indicates that the main force driving the peptides across the UFMs is the applied voltage (Bargeman *et al.,* 2002). In both A^-_{RC} and C^+_{RC} it was the PVDF in-situ experiments that showed the highest migration suggesting that when the conductivities of the membranes are right PVDF membranes performed better than PES for peptide migration. For instance UFM-I used in PVDF in-situ had a conductivity of 5.23 ± 0.04 mS/cm which is slightly less than the PES UFM-I used in PES in-situ experiment (5.94 ± 0.19), and yet we see a significantly higher final peptide migration for the PVDF in-situ (49.97 ± 6.25 µg/ml) compared to the PES in-situ experiment (17.92 ± 4.70 µg/ml) for A^-_{RC}. Similar differences were observed for the C^+_{RC} where significantly higher final peptide migration for the PVDF in-situ (53.86 ± 3.32 µg/ml) compared to the PES in-situ experiment (2.29 ± 0.39 µg/ml) (figure 4.7 & 4.8). Previous reports regarding UFM conductivity used in EDUF were focused on the use of conductivity as a follow up of fouling (Doyen A., *et al*. 2011), the possible link between UFM conductivity and peptide migration we reported here could be an area of future extensive investigation.

In EDUF experiments running for longer than 2hrs, for example as in the one reported by Doyen *et al.,* (2013) which ran for 4 hrs, peptide migration to the A^-_{RC} and C^+_{RC} increased in a curvy-linear fashion with a plateau attained after 180 minutes and 90 minutes , respectively. Doyen *et al.* (2013) also reported a higher peptide migration to both recovery compartments compared to the one we observed at final times. These differences could be because of the higher electric field strength they used, 14V/cm, compared to the 8.22V/cm applied in our experiments and also the 2hrs difference in EDUF duration.

4.2. Studies with PVDF Membranes Complimented with HPLC-MS

4.2.1. Membrane Characterization

4.2.1.1. Membrane thickness and conductivity

Table 2 below presents the thickness and conductivities of UFMs and IEMs used in the second part of our experiment. ANOVA showed no significant difference in thickness between UFM-I and UFM-II or between before and after EDUF. The thicknesses of IEMs varied significantly ($P<0.001$) between AEM and CEM but not between before and after EDUF. Conductivities of both UFM-I and UFM-II significantly changed before and after EDUF ($P<0.005$, in both cases) so did the conductivities of both IEMs change significantly before and after use in EDUF ($P<0.001$).

Table 2: Thickness (mm) and conductivity (mS/cm) of PVDF and IEMs before and after use in EDUF

	Thickness [mm]		**Conductivity [mS/cm]**	
Membrane	**Before EDUF**	**After EDUF**	**Before EDUF**	**After EDUF**
UFM-I	0.246 ± 0.002^{a}	0.248 ± 0.002^{a}	6.07 ± 0.27^{a}	5.65 ± 0.13^{b}
UFM-II	0.242 ± 0.002^{a}	0.245 ± 0.003^{a}	5.47 ± 0.36^{a}	5.08 ± 0.06^{b}
AEM	0.140 ± 0.001^{a}	0.140 ± 0.002^{a}	10.72 ± 0.89^{a}	8.68 ± 0.58^{b}
CEM	0.168 ± 0.003^{b}	0.172 ± 0.002^{b}	9.45 ± 0.91^{a}	7.39 ± 0.55^{b}

a and b indicate statistically significant difference ($P=0.005$) in a row

Generally the two UFMs had a close conductivity before use in EDUF (the differences are statistically in-significant) and this helps to avoid possible interference of membrane conductivity on peptide migration as indicated in section 4.1.3. The changes in thickness and conductivity are similar to the changes exhibited by PVDF membrane used in the first part of our experiment. But the changes in UFM conductivity used in the second part of our experiment are not as drastic as the ones used in the first part. This could be due to the relatively higher conductivity of the original PVDF membranes used in the second part which are indicative of healthier membranes which are less prone to fouling as suggested by Donose *et al*. (2011). The changes in IEM conductivity are similar to what was reported by other researchers (Casademont C., *et al.*, 2010) and are indicative of the possible deposition of peptides, amino acids and minerals onto the membrane causing it to foul and hence reducing its electrical conductivity.

4.2.1.2. UFM Zeta Potential

The zeta potentials of the UFM-I, UFM-II and the original PVDF membrane as a function of pH are presented in figure 4.9. All he membranes showed a positive zeta potential for pH range 2.5-4 while in the remaining pH range all of them showed a negative zeta potential. Zeta potential

generally decreased in the pH range 3-6, it remained relatively the same in the pH range 6-9 and generally decreased after pH 9 (figure 4.12). However significant differences were observed at extreme pHs: near pH 2.5-3.0 the native PVDF membrane exhibited a higher positive zeta potential compared to both UFMs while in the same pH range the UFM-I had the lowest positive (and even negative) zeta potential. The zeta potential recorded for UFM-II in this pH range were of an intermediate value of between the native and UFM-I (figure 4.9).

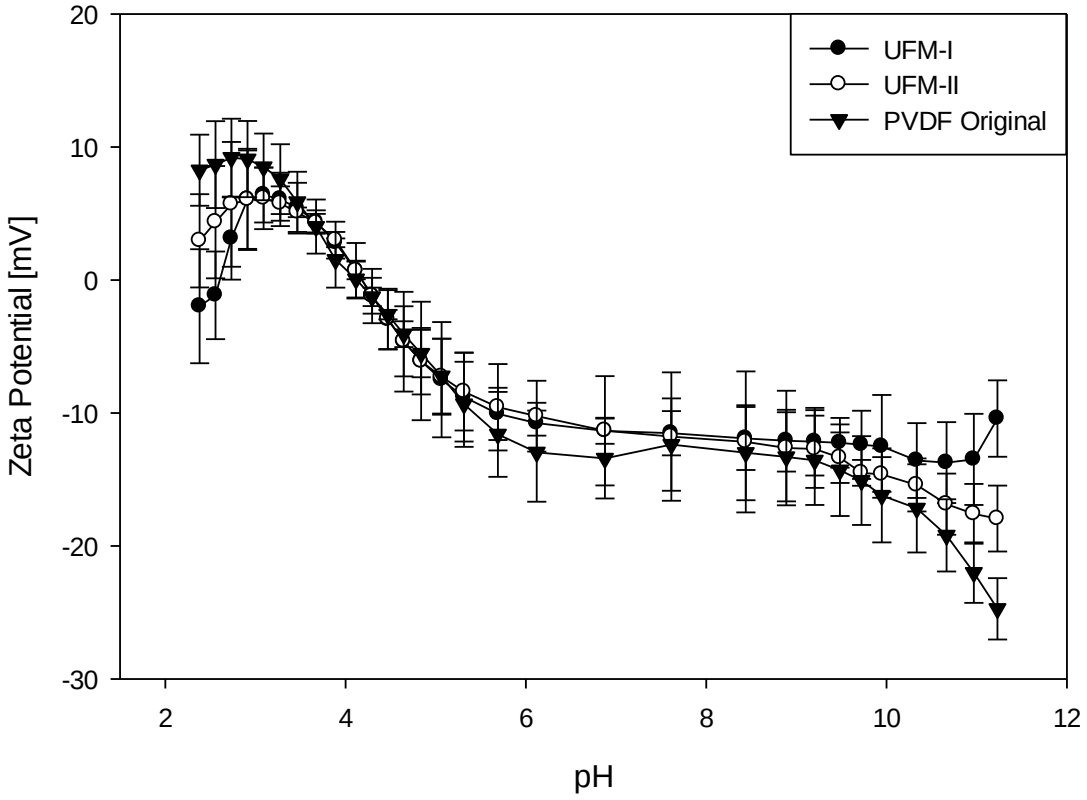

Figure 4.9: Zeta Potential of UFM-I and UFM-II (after use in EDUF) and the original PVDF membrane before use in EDUF

Even though the three membranes showed very close, similarly decreasing, zeta potential for the pH range 3.5 to 9.5, after pH 9.5 differences started to emerge. Here again the native PVDF membrane exhibited the largest negative zeta potential than both UFM-I and UFM-II. UFM-I showed a significant decrease ($P<0.005$) (in absolute value) of zeta potential compared to the original membrane while UFM-II exhibited a decrease (in absolute value) of zeta potential compared to the original membrane, the changes are less drastic compared to UFM-I.

The significant changes in zeta potential exhibited by UFM-I at extreme pHs as compared to the native PVDF membrane are indicative of the significant fouling (Al-Amoudi *et al.*, 2007) exhibited by this membrane after use in EDUF. The difference in the extent zeta potential reduction between UFM-I and UFM-II could be due to the fact that there are more anionic peptides generated than cationic ones leading to more fouling in UFM-I than UFM-II which is exposed to the relatively fewer peptides than the UFM-I.

4.2.2.EDUF Parameters

4.2.2.1. Evolution of system temperature and resistance

The evolution of system temperature and resistance during 120 min of EDUF for ex-situ and in-situ experiments is presented in Figure 4.10. System temperature increased from initial values close to room temperature (28^0C-30^0C) to 35^0C within the first 60 minutes of EDUF and the temperature was maintained at values close to 35 for the rest of the duration of EDUF making the use of external system of temperature controller a non necessity. ANOVA indicated no significant relation between ex-situ/in-situ digestions with system temperature change from initial values to final. The average temperature for ex-situ experiments was 33.32 ± 1.13^0C and for in-situ experiments was $34.55 \pm 1.35\ ^0$C.

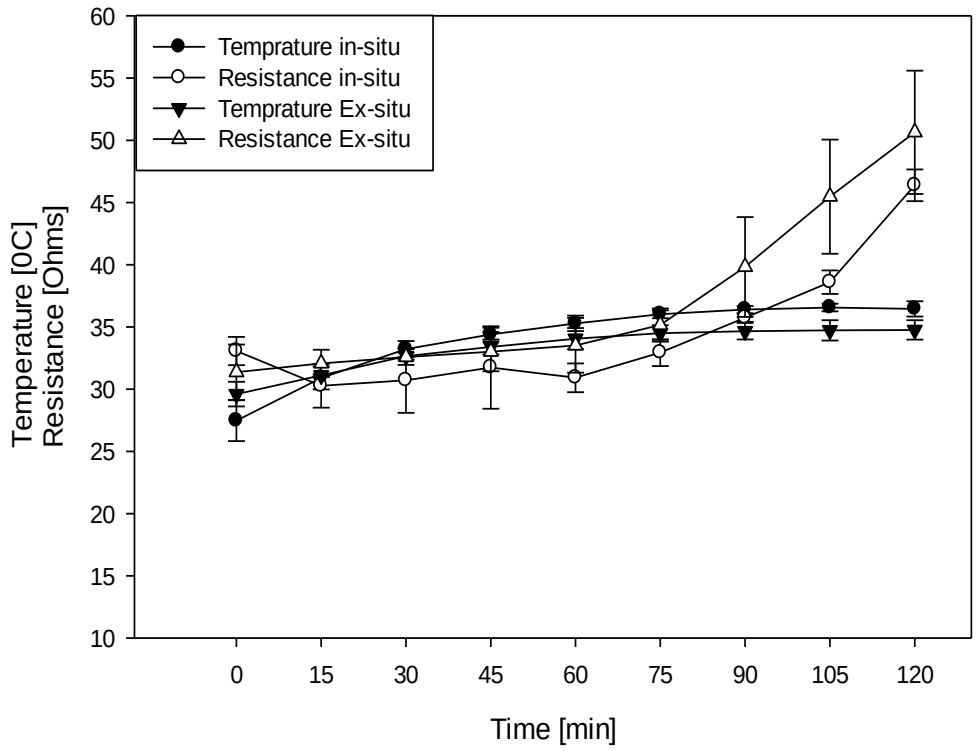

Figure 4.10: Evolution of system temperature and resistance as a function of time for ex-situ and in-situ experiments

System resistance evolved in a similar fashion as a function of time for both digestion strategies used as shown in figure 4.7. Initial slight decrease in resistance for the first 15 minutes of EDUF was followed by a relatively constant resistance for the next 60 minutes which is followed by a sudden rise in the system during the last 45 minutes of EDUF treatment. ANOVA indicated a significant difference (P=0.005) in system resistance evolution with time for the ex-situ and in-situ experiments: the ex-situ experiment exhibiting a consistently higher resistance than the in-situ one throughout the 120 minute EDUF except during the first 15 minutes of EDUF. In the ex-situ experiment system resistance varied from initial value of 31.36 ± 2.23 Ω to a final value of 50.65 ± 4.96 Ω while in the in-situ experiment it varied from 33.07 ± 1.135 Ω to 46.40 ± 1.27Ω. As mentioned in section 4.1.2 of part I the increasingly lower concentration of K^+ and Cl^- ions in the A^-_{RC} and C^+_{RC}, with time, due to their net migration into the feed and electrolyte compartments could be the reason for the increase in system resistance specially at final times of EDUF.

4.2.2.2. Evolution of conductivity in Different Compartments

Figure 4.11 depicts the change of compartment conductivity with time in the A^-_{RC} with time. Ex-situ and in-situ experiments had statistically insignificant different evolution of conductivity with time, with the A^-_{RC} in the in-situ having a slightly higher conductivity than the ex-situ. A similar evolution of compartment conductivity for the ex-situ and in-situ experiments was also observed in the C^+_{RC} (figure 4.12.) again ANOVA indicated a non-significant difference for ex-situ vs in-situ experiments. Average extent of demineralization in A^-_{RC} experiments was calculated to be 66.67 ± 5.12 % in the ex-situ experiment while it was slightly (statistically insignificant) lower at 64.90 ± 1.50 % in the in-situ experiment. For the C^+_{RC} the demineralization rate for both ex-situ and in-situ experiments averaged at 44.99 ± 4.7 %.

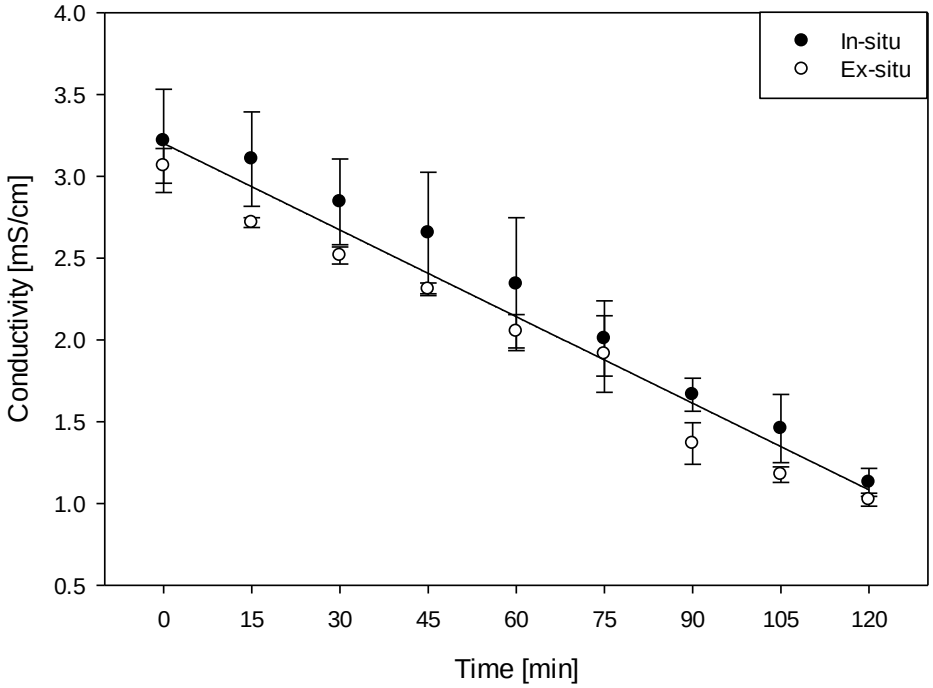

Figure 4.11: Evolution of conductivity as a function of time in the A^-_{RC} for ex-situ and in-situ experiments

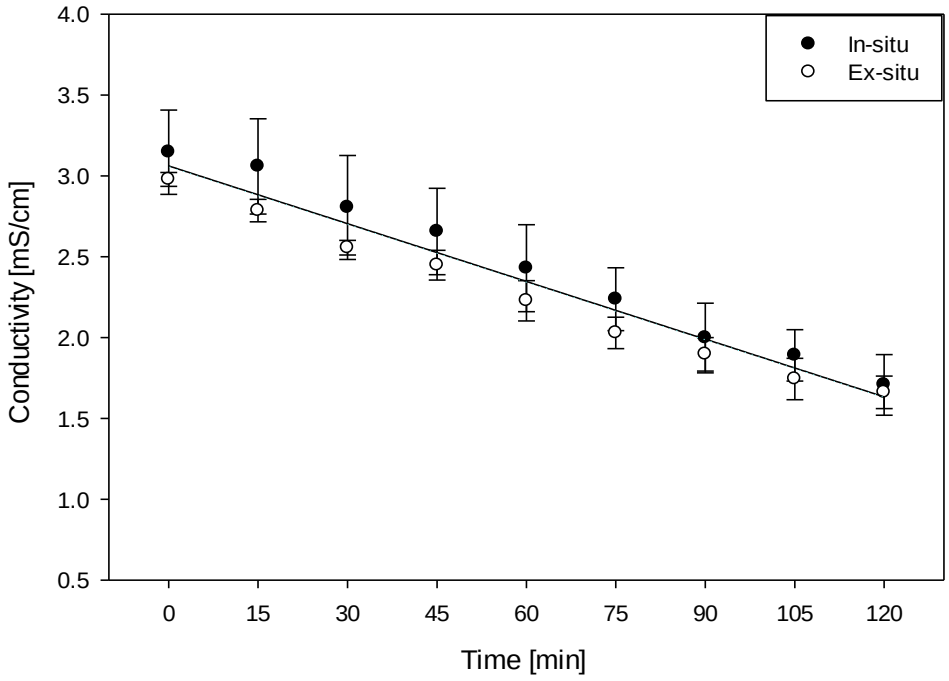

Figure 4.12: Evolution of conductivity as a function of time in the C^+_{RC} for ex-situ and in-situ experiments

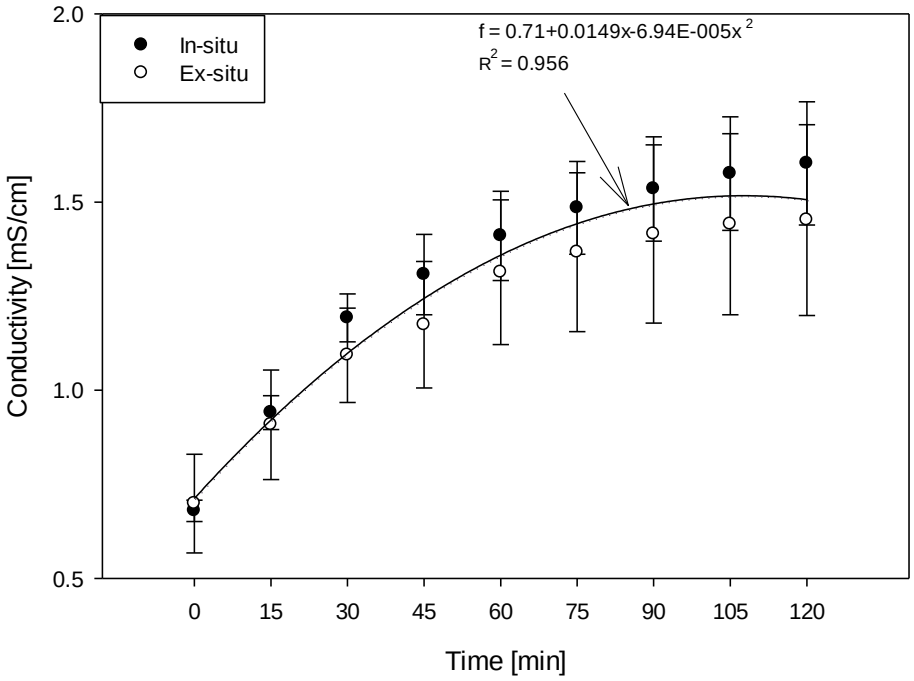

Figure 4.13: Evolution of conductivity as a function of time in the feed compartment for ex-situ and in-situ experiments

Similar to what was indicated in the first part of our result and discussion while the conductivities in A^-_{RC} and C^+_{RC} decreased with time the conductivity in the hydrolysate compartment increased with time (Figure 4.13) for part II as well. The increase was more curvilinear than just linear as indicated by the corresponding quadratic equation on the fitted curve. ANOVA indicated no significant difference between the in-situ and ex-situ experiments with respect to change in conductivity in the hydrolysate/ feed compartment.

The decreases in conductivity in the A^-_{RC} was due to the transfer of K^+ and Cl^- ions from the A^-_{RC} into the hydrolysate and electrolyte solutions on the cathode side, respectively. K^+ and Cl^- ions also transferred from the C^+_{RC}, respectively, into the electrolyte solutions on the anode side and into the hydrolysate solution (Figure 3.1). The anions and cations (especially, Na^+, OH^- coming from pH adjustment and also the peptides) present in the hydrolysate migrated, to the electrolyte solutions near the anode and cathode sides, respectively, but as reported by Poulin *et al.*, (2006) there is a net transfer of K^+ ions migrating from the A^-_{RC} and Cl^- ions migrating from the C^+_{RC} leading to the observed mineralization in the hydrolysate compartment.

4.2.2.3. Evolution of Total Peptide Migration

Peptide migration into both cationic and anionic compartments for both ex-situ and in-situ experiments are presented in figure 4.14. ANOVA indicated a significant dependence of peptide migration on time of EDUF ($P<0.0001$), recovery compartment (A^-_{RC} or C^+_{RC}, $P<0.005$), and also on ex-situ/in-situ digestion ($P<0.001$). Migrations to C^+_{RC} in both digestion strategies increased in a manner that attained a plateau at 90 minutes of EDUF (figure 4.14). Peptide migration to the A^-_{RC} appeared to continue increasing beyond 120 minutes (figure 4.14). These observations are similar to the ones reported by Doyen *et al* (2013).

The peptide concentration in the anionic recirculation compartment (A^-_{RC}) was significantly lower ($P<0.001$) for the ex-situ experiment than the in-situ throughout the 120 minutes of EDUF with respective final values of 54.17 ± 1.18 µg/mL and 77.93 ± 13.02 µg/mL. This observation is similar to the observation made for PVDF ex-situ experiment in part I of our experiment (section 4.2-I). This suggests that even when the conductivity of the original UFM-I used was not very low the ex-situ experiment generally led to a lower migration than the in-situ experiment with regards to the A^-_{RC}. This could be because the ex-situ experiment allowed time for possible peptide-peptide interaction that could happen between the relatively large number of anionic peptides that were generated leading to possible loss of charge and gain of mass which reduce migration. In the in-situ experiment the chances for the peptides to interact were relatively limited because separation happened simultaneously with digestion.

It was observed that, for the duration of the EDUF in our experiment, migration to the C^+_{RC} appeared to be higher than migration to the A^-_{RC} (in both ex-situ and in-situ digestions figure 4.14). This could be because of the 2hrs duration of the EDUF during which the migration to the A^-_{RC} had not attained its maxima as opposed to migration to the C^+_{RC} which had attained a plateau after the 2hrs of EDUF (figure 4.14). In a 4 hour EDUF experiment using a similar setup but a different UFM material (Cellulose Acetate-CA) and a 60V applied voltage, Doyen *et al.*, (2013) reported a statistically significant higher migration to the A^-_{RC} as compare to C^+_{RC} which is in agreement with our hypothesis that, if enough time is allowed, final migration to the A^-_{RC} will be higher than migration to the C^+_{RC}.

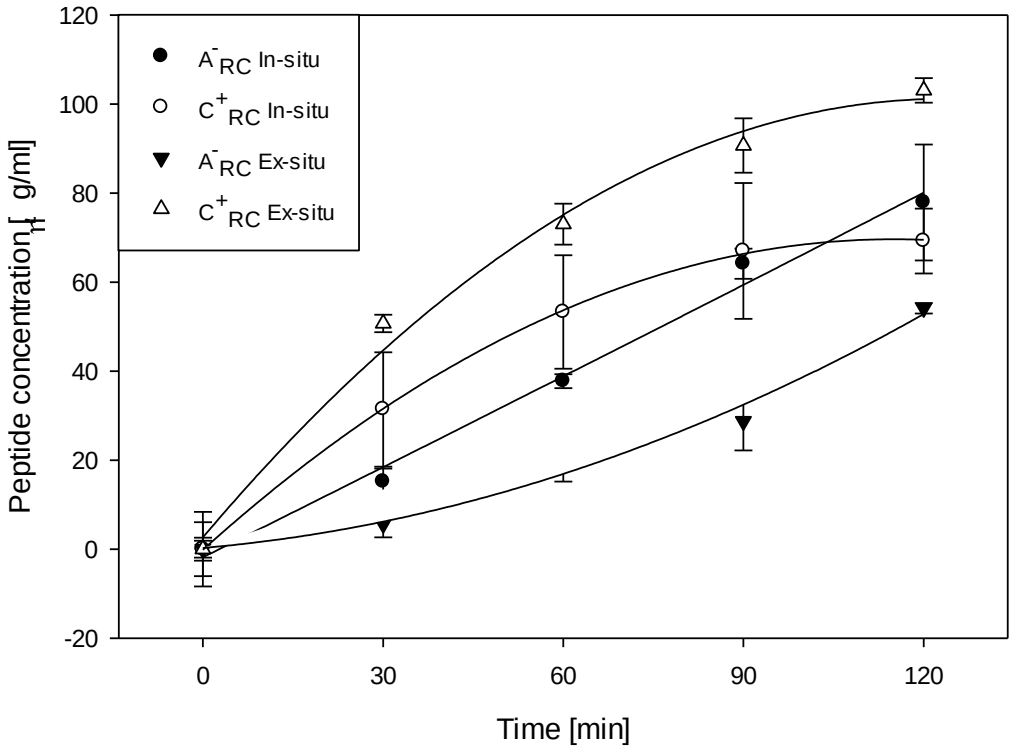

Figure 4.14: Evolution of total peptide concentration in the A^-_{RC} and C^+_{RC} as a function of time for ex-situ and in-situ experiments with PVDF

This difference in migration could be because of the higher anionic peptides generated in trypsic hydrolysis of BiPro (or β-LG), at pH 7.8, than cationic peptides (Groleau *et al.*, 2003).

For the C^+_{RC} a larger final peptide migration was recorded for the ex-situ experiment (103.10 ± 2.76 µg/mL) compared to the in-situ (69.26 ± 7.29µg/mL). This observation is different from the one observed for the A^-_{RC} in which in-situ led to a higher migration than ex-situ. This could be because there is less significant effect of peptide-interaction amongst cationic peptides as mainly hydrophobic, not electrostatic/ionic, interactions are involved aggregation process of peptides produced from trypsic digestion of milk proteins (Groleau *et al.*, 2003). The hydrophobic interaction amongst cationic peptides (or cationic peptides with other peptide) are relatively limited because of their lesser quantities compare to the larger quantities of anionic peptides making the cationic peptides less affected by ex-situ digestion.

For the in-situ experiments in part-I and part-II close values of final peptide migration to the C^+_{RC} were recorded, 53.55 ± 3.77 µg/ml and 69.26 ± 7.29 µg/mL respectively. The migrations to the C^+_{RC} during the ex-situ experiments were markedly different from each other in part-I and part-II with respective values of 16.56 ± 5.36 µg/mL and 103.10 ± 2.76 µg/mL. This could be

due to the marked difference in conductivities of the two UFM-IIs used in part-I and part-II (2.73 ± 0.32 mS/cm and 5.47 ± 0.56 mS/cm, respectively) as proposed previously.

4.2.3. Peptide Profiles

4.2.3.1. Peptide Profile in the Hydrolysate Compartmentff

Major peptides obtained from 120 minutes of digestion of BiPro by trypsin with their respective HPLC retention time, mass, potential sequence, pI and possible source native protein are presented in table 3. Potential sequences, peptide location, pI and net charges were obtained by tools from the ExPAsy Bioinformatics Resource Portal (Swiss Institute of Bioinformatics) for bovin (Bos taurus) β-LG (UniProtKD/TrEMBL # P02754), Lactoperoxidase (UniProtKD/TrEMBL # P80025), Lactoferin UniProtKD/TrEMBL # B9VPZ5) BSA (UniProtKD/TrEMBL # P02769) α-lactalbumin precursor (UniProtKD/TrEMBL # P00711) and immunoglobulin gamma Fc region receptor II precursor (IgG Fc receptor II-UniProtKD/TrEMBL Q28110) digestion by trypsin. These proteins along with other minerals, sugars and amino acids are indicated to be present in BiPro by the manufacturer (DAVISCO). The peptide fragments labeled Nd in table 3 are not generated by the ExPAsy Bioinformatics Resource server and it is assumed that these peptides may be generated by peptide-peptide interactions that have also been observed before (Doyen A., *et al.*, 2011).

Of the 23 peaks listed on table 3, nine were negatively charged (anionic, peak # 1, 2, 3, 5, 11, 16, 17, 22, 23), three positively charged (cationic, peak # 6, 7 & 14) at the pH of operation (7.8) while 6 (Peak # 4, 12 ,15, 18, 20, 21) did not have a corresponding peptide generated by ExPAsy Bioinformatics Resource Portal (hence Nd). Five of the peaks (peak # 8, 9, 10, 13 and 19) contained more than one possible peptide which were either anionic, cationic or Nd. Similar to our observation mentioned above trypsic hydrolysis of milk proteins have previously been reported to generate more anionic peptides than cationic ones (Groleau *et al.*, 2003).

Table 3: **Characterization of peptides obtained after 120 minutes of EDUF**

Peak #	R_t (min)[a]	Obs MW[b]	Potential Sequence [c]	Location [d]	Net Charge [e]	pI[f]	Source of Peptide [g]
1	11.442	573.4	IIAEK	f71-75	-	6	β-LG
2	24.865	916.4	IDALNENK	f84-91	-	4.4	β-LG
3	28.279	673.3	GLDIQK	f9-14	-	5.9	β-LG
4	29.172	949.5	Nd [h]	Nd	Nd	Nd	Nd
5	31.298	1245.0	TPEVDDEALEK	f125-135	-	3.8	β-LG
6	35.282	933.5	LIVTQTMK	f1-8	+	8.8	β-LG
7	36.63	837.5	ALPHMIR	f142-148	+	9.8	Nd
8	38.151	1193.7	VLVLDTDYKK	f92-101	-	5.9	β-LG
		837.5	ALPHMIR	f142-148	+	9.8	β-LG
		1437.6	Nd	Nd	Nd	Nd	Nd
9	39.61	903.8	TKIPAVFK	f76-83	+	10	β-LG
		1635.9	TPEVDDEALEKFDK	f125–138	-	4	β-LG
10	40.272	696.3	VAGTWY	f15-20	-	5.49	β-LG
		697.4	KPDLPK	f43-48	+	10.9	Ig-ɣ[i]
		1635.9	TPEVDDEALEKFDK	f125–138	-	4	β-LG
11	41.107	1065.6	WENGECAQKK	f61-70	-	6.1	β-LG
12	43.697	2163.0	Nd	Nd	Nd	Nd	Nd
13	44.735	1163.7	LVNELTEFAK	f66-75	-	4.5	BSA
		1361.1	GSNFQLDQLQGR	f120-131	-	5.8	β-LG
14	45.76	1200.7	VGINYWLAHK	f118-127	+	8.6	α-LAP[k]
15	48.396	2309.0	Nd	Nd	Nd	Nd	Nd
16	52.816	2030.2	SLAMAASDISLLDAQSAPLR	f21-40	-	4.21	β-LG
17	53.913	1157.0	LICDNTHITK	f669-678	-	6.7	Lpx [j]
18	56.545	1185.0	Nd	Nd	Nd	Nd	Nd
19	57.421	1477.8	LGEYGFQNALIVR	f421-433	-	6	BSA
		1567.7	DAFLGSFLYEYSR	f347-359	-	4.4	BSA
		1152.0	Nd	Nd	Nd	Nd	Nd
20	59.617	1443.7	Nd	Nd	Nd	Nd	Nd
21	60.179	1269.0	Nd	Nd	Nd	Nd	Nd
22	61.196	1301.6	ETTVFENLPEK	f230-240	-	4.3	Lactoferrin
23	65.912	2707.4	VAGTWYSLAMAASDISLLDAQSAPLR	f15–40	-	4.2	β-LG

[a] Retention time [b] Observed Molecular Weight [c,d,e,f,g] According to ExPAsy Bioinformatics Resource Portal,

[h] Non-defined, [i] Immunoglobulin gamma, [j] Lactoperoxidase, [k] Alpha Lactalbumin

Figure 4.15 depicts the chromatograms of BiPro solution before digestion by trypsin. Peaks labeled A and B with retention time 70.249 and 72.293, respectively, stand out in the HPLC chromatogram and correspond to proteins identified as β-LG A and β-LG B, the two major variants of β-LG protein in milk with respective molecular mass of 18,362.86 Da and 18,276.73 Da (Brownlow S. *et al.*, 1997). Manufacturer (DAVISCO) indicated that the other proteins present in BiPro all together constitute to less than 5% by mass and that could be why they are not visible on the chromatograms relative to the 95% β-LG composition of BiPro.

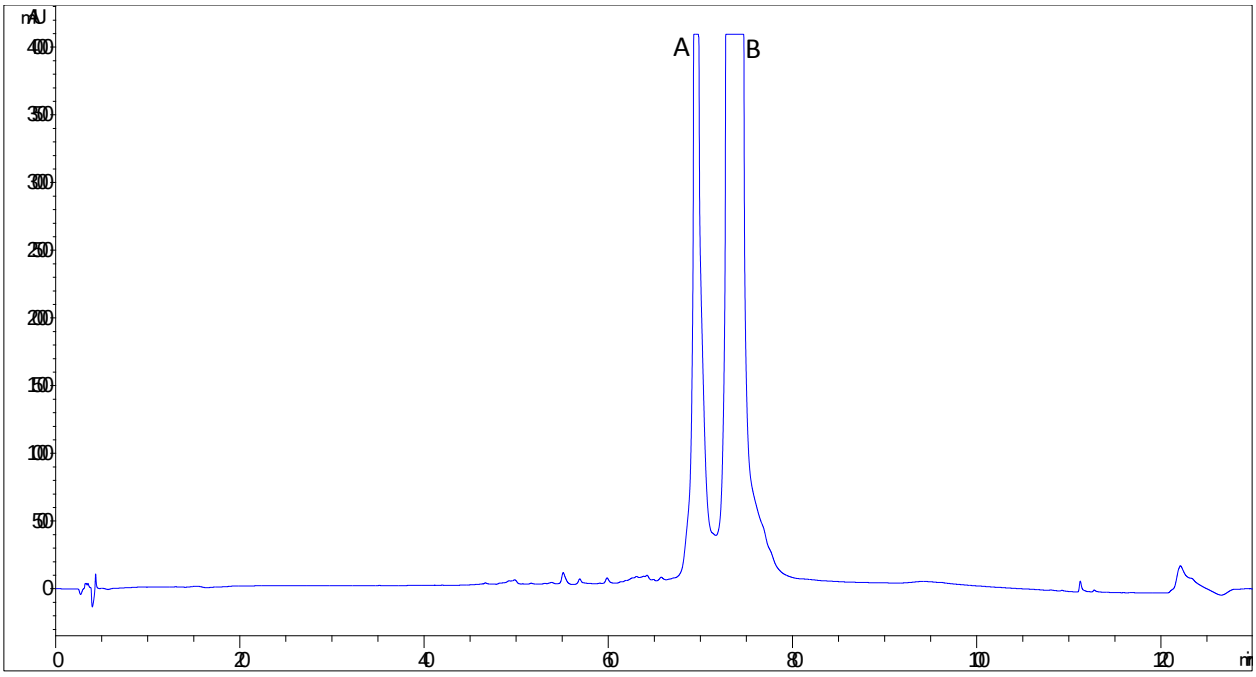

Figure 4.15: Chromatogram of initial BiPro solution before digestion by trypsin

Figure 4.16 A, B, and C depict typical chromatograms of BiPro after 120 minutes of ex-situ digestion, BiPro after 120 minutes of EDUF after an ex-situ digestion and after 120 minutes of simultaneous (in-situ) digestion and EDUF treatment of BiPro, respectively. A comparative analysis of chromatograms A and B of figure 4.16 illustrates the effect of EDUF treatment on the individual peaks (peptides). As it can clearly be seen on the two chromatograms (A& B, fig 4.16) almost all the peaks in B appeared reduced in heights, and hence areas, as compared to their corresponding peaks in A (ex-situ-beaker digestion). This was expected and was because of the migration of the peptides from the feed compartment to the A^-_{RC} and C^+_{RC} in B, but such migration was not happening in A. The same trend of peak area decrease was observed when comparing respective peaks in A and C (Figure 4.15). In C (in-situ digestion and EDUF) the peptide migration was happening at the same time the peptides were being generated by hydrolysis and it was again expected that peaks in C would be smaller than the ones in A. Generally speaking B and C presented similar chromatograms.

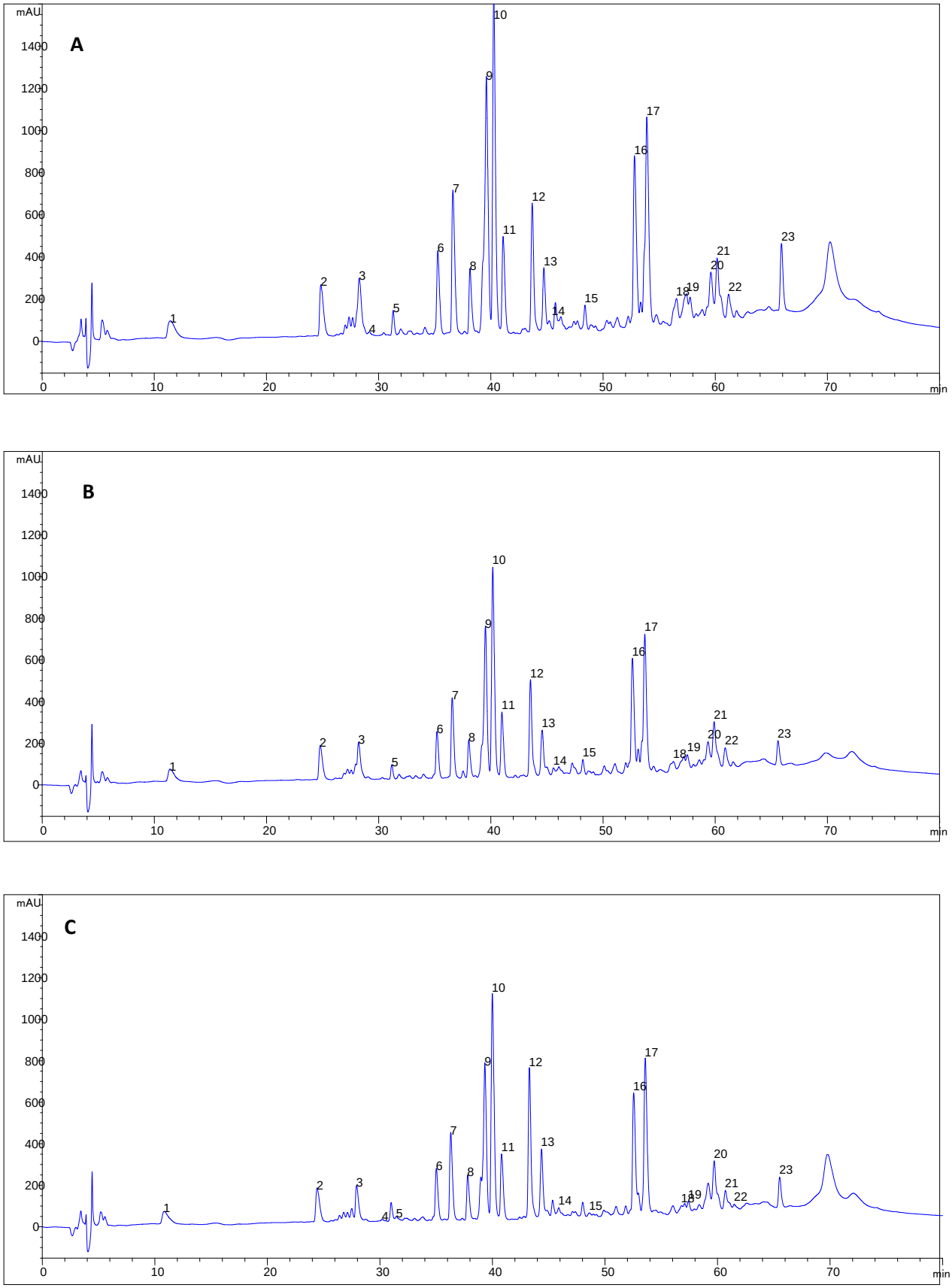

Figure 4.16: Chromatograms of BiPro A) after 120 minutes of ex-situ digestion, B) after 120 minutes of EDUF after an ex-situ digestion and C) after 120 minutes of simultaneous (in-situ) digestion and EDUF treatment

The dilution caused by the dead volume of the ED unit, which is 0.5L, could also contribute to the difference in the peak areas observed above.

4.2.3.2. Peptide Profiles in the A^-_{RC} and C^+_{RC}

Figure 4.17 below display typical HPLC chromatograms of the peptide fractions obtained in the A^-_{RC} after 120 minutes of ex-situ and in-situ EDUF. In both cases peak numbers # 5 and 9 migrated to a marked extent relative to the other peaks and in the ex-situ experiment (Fig 4.17 A) these two peaks appeared slightly of higher area, and hence quantity, than in the in-situ experiment (Fig 4.17: B). Peak # 2 and 17 showed a relatively similar pattern in both cases but again the ex-situ chromatograms appear slightly of a larger area than the in-situ ones for these peaks. In general, for the A^-_{RC}, peaks in ex-situ experiment were observed to be of larger area compared to their corresponding peaks in the in-situ experiment (Figure 4.17 A & B).

Peak # 8 which exhibited a marked migration in the ex-situ experiment (Fig 4.17 A) is totally missing from the A^-_{RC} of the in-situ experiment. Peak # 8 was composed of 3 peptides (with a positive, a negative and a non-defined charge-Table 3) which could be the reason why it exhibited such a complex pattern of migration. Moreover in the in-situ experiment a peak with retention time 33.578 minutes was observed to have a marked migration while such a peak was almost negligible, and hence not picked up by the HPLC Agilent Chemstation Software used to integrate the peaks, in the ex-situ experiment.

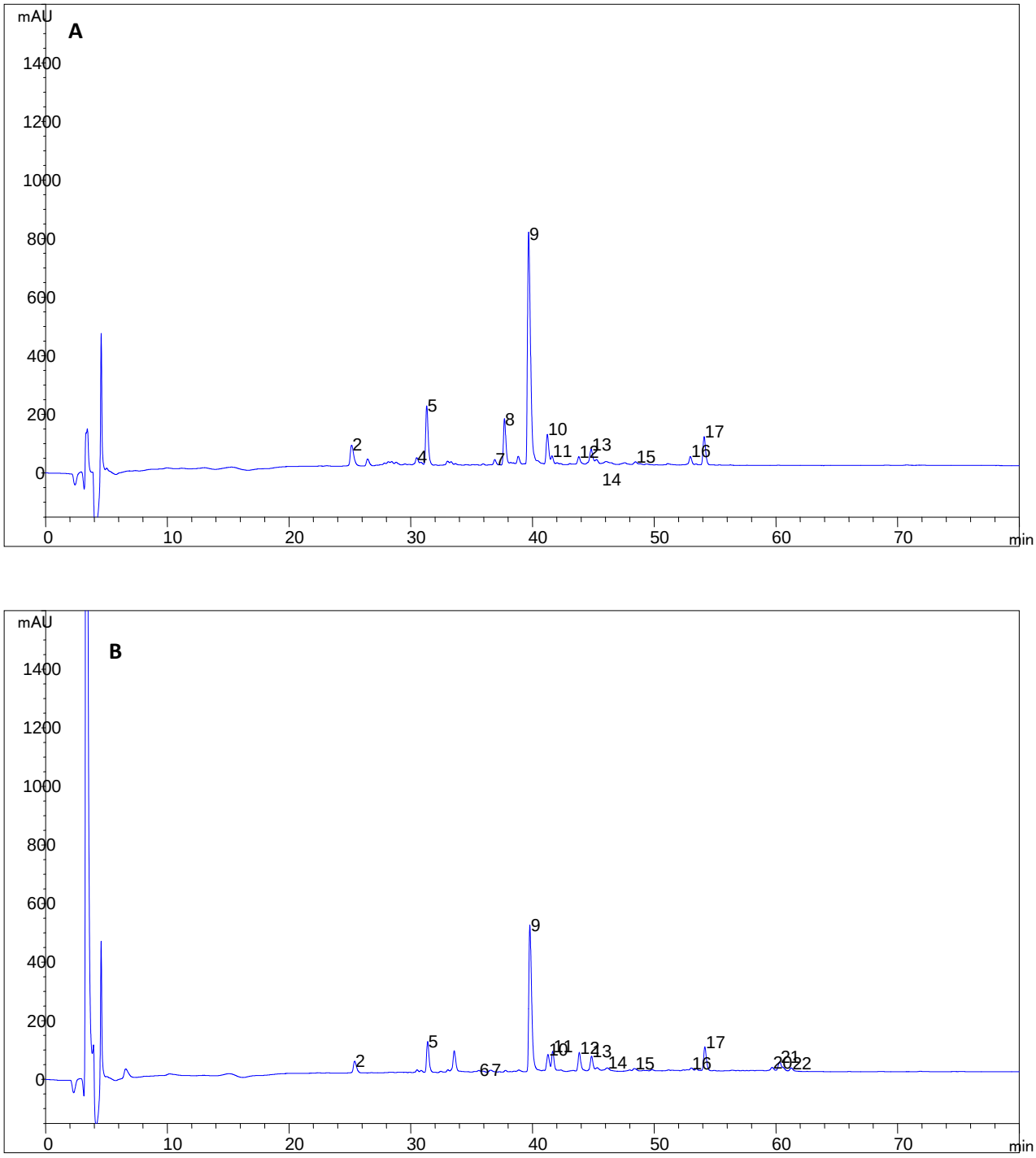

Figure 4.17: Chromatograms of peptides obtained in the A^{-}_{RC} after 120 minutes EDUF of A) ex-situ digested BiPro and B) in-situ digested BiPro

MS identified a peptide of mass 1094.8 Da at this retention time but, according to ExPAsy Bioinformatics Resource Portal, no such peptide with a negative charge, at pH 7.8, was generated from the proteins contained in BiPro when digested by trypsin. However a closer look at one of the peptides at peak #8 give a possible explanation for the loss of this peak and

appearance of another peak in the in-situ digestion. Peak #8 contains a peptide with mass 1193.7 Da and sequence VLVLDTDYKK and a loss of one valine amino acid (MW 117.1 Da) from the N-terminal and accompanied by addition of water (MW 18 Da) would give a peptide of mass 1094.6 Da which is extremely close to the peptide detected only in the in-situ digestion. It is interesting to note here that the peptide detected in the ex-situ digestion (MW 1193.7 Da) is reported to be an antimicrobial peptide (Pellegrini *et al*., 1997) while there is no apparent bioactivity reported for the peptide obtained in the in-situ digestion.

Differences in migration of specific peptides were different from the peptide migration observed by the BCA method for the A^-_{RC} (reported in section 4.2.2.3) where it was observed that there was a significantly higher ($P<0.005$) total peptide migration in the in-situ experiment compared to the ex-situ experiment. This could be because the BCA measures total peptide migration including peptides with smaller than 500Da and higher than 3000Da which are excluded from the HPLC-MS by the method we used.

Typical chromatograms for C^+_{RC} fractions obtained after 120 minute of EDUF after ex-situ digestion and after in-situ (simultaneous) EDUF and digestion are presented in figure 4.18 A & B, respectively. In general the peptide peaks obtained for the ex-situ experiment (Figure 4.18 A) appear to be of higher areas than the ones in the in-situ experiment. This is similar to the observation made for A^-_{RC} in which chromatograms for the ex-situ experiment had a slightly larger area than the in-situ experiment. In the case of total peptide migration to the C^+_{RC} estimated by the BCA method (section 4.2.2.3) it was observed that ex-situ setup led to a higher migration than the in-situ setup which is in agreement to the general observation made from the chromatograms on figure 4.18.

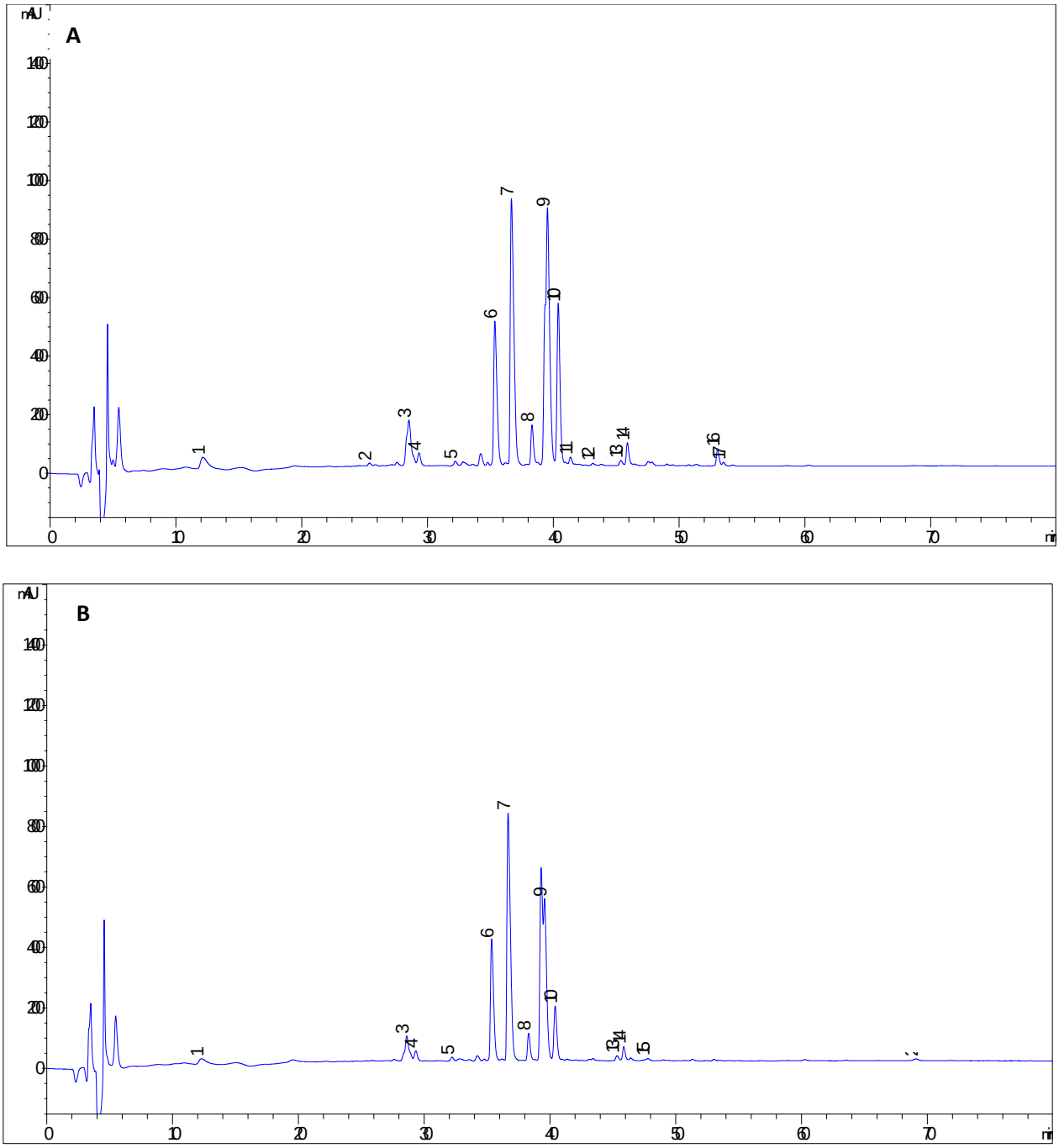

Figure 4.18: Chromatograms of peptides obtained in the C^{+}_{RC} after 120 minutes EDUF of A) ex-situ digested BiPro and B) in-situ digested BiPro

Fewer cationic peptides than anionic peptides are generated by trypsic digestion of BiPro (Table 3) and this may explain the similarity observed for the estimates of peptide migration to the C^{+}_{RC} by the BCA method and HPLC-MS analysis as opposed to the difference observed for estimates of peptide migration to the A^{-}_{RC} by the two methods. The larger amounts of anionic peptides generated, and potentially migrated to the A^{-}_{RC} and were detected by the BCA method while the HPLC-MS method was more specific for a specified peak # accounting for the observed difference. The few cationic peptides that are generated, and potentially migrated, to

the C^{+}_{RC} are also detected by the HPLC-MS accounting for the similarity observed by the estimates.

Peak # 6 & 7 were markedly present in the C^{+}_{RC} for both ex-situ and in-situ experiments and this could be because of the positive charge they exhibited at the pH of operation (pI=8.8 for peak 6 and pI=9.8 for peak 7) and their relatively lower molecular mass (933.5 Da and 837.5 Da respectively). Peak # 9 and 10 also exhibited marked migration to the C^{+}_{RC} for both ex-situ and in-situ experiments and this could be because these peaks contain components that are positively charged at the pH of operation (7.8) and these cationic peptides happen to be of relatively lower molecular mass (903.8Da and 693.3 Da, respectively). However these peaks also contain other peptides with negative charges (1635.9 Da and 697.42 Da, respectively) and hence they are also observed in the A^{-}_{RC} markedly, possibly because of the lower pI value (4.0) for the peptide with mass 1635.9 Da and the combination of lower mass and a pI value of 5.49 for the peptide with a mass of 697.42 Da in peak #10.

The power of EDUF in separating peptides of close molecular weight is illustrated by a closer look at peak #9. One of the two peptides contained in peak#9 with molecular mass 903.8 Da is totally missing from MS of the A^{-}_{RC} while it was detected by MS in the C^{+}_{RC}. On the other hand the anionic peptide present in this peak (molecular weight 1635.9 Da) is totally missing from C^{+}_{RC} while it was detected in the MS of the A^{-}_{RC} sample. The peptide with molecular mass 903.8 Da has a sequence of TKIPAVFK and known to possess a hypocholesterolemic function (Nagaoka *et al.*, 2001). Other isolated bioactive peptides include peptide of peak #1 and peak #3 which are also hypocholesterolemic (Nagaoka *et al.*, 2001), the peptides from peak #2, 7 & 23 were identified as antihypertensive (Chobert, J., *et al*., 2005 & Mullally M., *et al.*, 1997). The anionic peptide in peak #8 is known for its anti-bacterial activity (Mullally M., *et al.*, 1997).

5. Conclusions and Future Perspectives

Peptide migration was found to be strongly dependent on ultrafiltration membrane (UFM) conductivity than its type and the digestion strategy used. Generally peptide migration to the A^-_{RC} is found to be higher than peptide migration C^+_{RC}. For UFMs with close values of conductivity, peptide migration to the A^-_{RC} was observed to be higher with in-situ digestion while peptide migration to the C^+_{RC} was higher in an ex-situ digestion. Peptide migration to the C^+_{RC} varied from 16.56 ± 5.36 µg/mL to 103.10 ± 2.76 µg/mL for PVDF membranes with significantly different conductivities: 2.73 ± 0.32 mS/cm and 5.47 ± 0.56 mS/cm, respectively. When the two membrane types, PES and PVDF, had closer values of conductivities PVDF was observed to exhibit more migration than PES for the same strategy of digestion. For instance, PES in-situ experiment with UFM-I (forming the barrier between feed and A^-_{RC}) of conductivity 5.94 ± 0.19 mS/cm led to migration of 22.94 ±4.46 µg/ml while PVDF in-situ experiment with UFM-I conductivity 5.23 ± 0.04 led to a migration of 49.65 ± 6.13 µg/ml. Peptide migration to the A^-_{RC} was higher in an in-situ digestion than ex-situ digestion while peptide migration to the C^+_{RC} was higher in an ex-situ setup than in-situ setup, regardless of membrane type. HPLC-MS studies identified 23 major peaks that were generated on whey protein isolate digestion by trypsin. The migration of these peaks to the A^-_{RC} or C^+_{RC}, or both, illustrated the power of EDUF in isolating bioactive peptides from complex mixtures that contain peptides with close mass/shape characteristics but with no apparent bioactivity. Among the anionic peptides 3 peptides are known to have hypocholesterolemic effect, 1 as antibacterial and 1 as antihypertensive. Among the cationic peptides 1 is reported to have an antihypertensive activity.

In light of the major results presented above it is the author's recommendation that UFMs be screened for difference in broad range of parameters than thickness, conductivity and zeta potential. Factors like surface roughness measured by AFM and SEM and membrane surface hydrophobicity determined by contact angle measurements could shade more light on the differences we reported here. Alternating ex-situ and in-situ experiments in between repeats could also be tested to avoid the interference of fouling.

To our knowledge this is the first report that shows a strong link between UFM conductivity and peptide migration during EDUF. Further studies in screening UFMs and possible correspondence with manufacturers to custom design UFMs with exceptionally high peptide migration and the study of these custom designed UFMs could be an interesting idea to take on. Moreover the difference in migration to the different recovery compartments reported here also needs further investigation depending on which compartment one is interested in. Though EDUF is attractive for preparative-scale separations, because it readily separates components of

a sample in space and in time, research in industry scale is lacking. To this end, a coupling of EDUF with pressure driven process can be investigated for increased efficiency.

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